CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF FUNGAL ENDOPHYTES FROM SELECTED KENYAN MEDICINAL PLANTS

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DECLARATION AND RECOMMENDATION

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This thesis is my original work and has not been submitted or presented for examination in any institution.

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DEDICATION

To my parents Mr. and Mrs. Everet Bradley Wekesa, for their unconditional love and constant support through this study.

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ABSTRACT

Infectious diseases remain to be a global health burden due to the development of antibiotic resistance by pathogenic microorganisms. Antibiotic resistance has led to increased number of deaths among children and adults. This study sought to screen for antimicrobial activity of extracts from fungal endophytes against selected human pathogens, identify, and characterize the fungal endophytes and screen for phytochemicals present in selected medicinal plants' extracts. Sixty-five fungal endophytes were successfully isolated from fresh leaves of twenty-two selected medicinal plants. The DNA of fungal endophytes was extracted using BIO BASIC EZ-10 spin column DNA miniprep kit, and molecular characterization was done using internal transcribed spacer region (ITS). Molecular identification revealed that all the fungal endophytes belonged to the Ascomycota group of fungi distributed in nine genera; *Fusarium*, *Colletotrichum*, *Trichothecium, Phomopsis*, *Pestalotiopsis*, *Cladosporium*, *Aspergillus*, *Phoma,* and *Chaetomium*. With endophytes in the genera *Colletotrichum* and *Fusarium* having the highest isolation frequency. Antagonistic assays demonstrated that 63.6% of the fungal endophytes showed antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Crude extracts of selected fungal endophytes exhibited antibacterial activity against *Bacillus subtilis* (27 mm), *S. aureus* (22 mm), *Escherichia coli* (25 mm), *P. aeruginosa* (12 mm) and *Klebsiella pneumoniae* (12 mm). Extracts from *Aspergillus* sp. demonstrated antimicrobial activity at low concentrations of 2.34µg/ml against *B. subtilis* and 9.38µg/ml against *Candida tenius* in the serial dilution assay. Phytochemical screening of leaf extracts revealed the presence of terpenoids, phlobatannins, flavonoids, tannins, and saponins. These results show that medicinal plants are a reservoir to fungal endophytes, which could be exploited as sources of natural products of pharmaceutical importance.

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LIST OF ABBREVIATION AND ACRONYMS

ASTM	American Society for Testing and Materials
ATCC	American Type Culture Collection
BCCM	Belgian Coordinated Collections of Microorganisms
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Unit
DMSO	Dimethyl sulfoxide
DSM	German Collection of Microbes and Cell Cultures
HSD	Honestly Significant Difference
ITS	Internal Transcribed Spacer
MIC	Minimum Inhibitory Concentration
NCBI	National Centre for Biotechnology Information
Nr DNA	Nuclear ribosomal Deoxyribonucleic acid
PDA	Potato Dextrose Agar
rDNA	Ribosomal Deoxyribonucleic acid
SDA	Saboraud Dextrose Agar
SM	Sugar-malt
TLC	Thin Layer Chromatography
YM	Yeast Malt

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background Information

Infectious diseases are the second major cause of deaths, according to the global burden of diseases World Health Organization (WHO) report (WHO, 2014). From the report, it is estimated that infectious and parasitic diseases cause about 18.4% deaths worldwide. This problem is linked to the development of multidrug-resistant pathogenic bacteria. Several research initiatives have shown results of drug resistance in human pathogenic bacteria from all over the world (Andremont, 2001; N'guessan *et al*., 2007; Lu *et al*., 2007; Mbwambo *et al*., 2007). As a result, extensive attention has been focused on finding alternative antimicrobial compounds from natural sources.

The problem of drug resistance is observed in many microbial pathogens, which are resilient and have developed several ways to resist antibiotics and other antimicrobial drugs. Additionally, the frequent use and misuse of existing antibiotics in human and veterinary medicine as well as in agriculture is another cause. Currently, over 70% of infections caused by bacteria in health care units present resistance to the commonly used drugs. Some of these microorganisms can only be treated with experimental and potentially toxic drugs. The emergence of multidrug resistance has been reported in various countries making therapeutic options limited (Song *et al*., 2004; Jenkins *et al*., 2005; Johnson *et al*., 2006; Zhou *et al*., 2011; Charfi *et al*., 2012). In a recent study, the prevalence of penicillin-resistant *Streptococcus pneumoniae* has been increasing worldwide (Jenkins *et al*., 2005; Hoban *et al*., 2005; Yang *et al*., 2008; Varon, 2012). Microbial development of resistance, as well as economic incentives, has resulted in the search for new and effective antibiotics. The development of resistant strains is unavoidable, but the ways that antibiotics are administered and used have greatly exacerbated the problem. Unless we take into consideration the problem of antibiotic resistance as they emerge, the danger posed by previously treatable diseases will continue to lurk as previously manageable health problems re-emerge.

Natural products play a significant role in the discovery of leads for the development of pharmaceuticals for the treatment of human diseases. Medicinal plants are used in the treatment

of diseases in Africa and the developing world (Phillipson, 1994). They accumulate valuable secondary metabolites as a means of surviving in a hostile environment (Manach, 2004) hence; they have been used in folk medicine (Calixto, 2000). Medicinal plants are also a host to endophyte diversity that produces novel bioactive natural products of pharmaceutical significance (Tan & Zou, 2001; Strobel *et al*., 2004). Endophytes are a group of microorganisms that reside in the plant tissues without causing any damage to the host plant (Stone *et al*., 2000). Currently, they are viewed as a rich source of bioactive compounds (Weber *et al*., 2007). Therefore, they make a vast and yet a significant untapped source of new pharmaceuticals. Despite the fact that a lot has been documented on antibiotic resistance, the problem is still persistent and this is compounded by the fact that the development of antimicrobial compounds has remained slow. This study aimed to isolate and identify fungal endophytes from leaves of selected medicinal plants, which can then be used for the discovery of potentially new drugs.

1.2 Statement of the Problem

According to the World Health Organization reports, infectious diseases have time been among the leading cause of deaths in human population affecting both children and adults. This is attributed to the rise in antibiotic resistance by disease-causing microorganisms. The cause of antibiotic resistance has been linked to the inappropriate use and misuse of antibiotics by a greater proportion of the entire human population. The global health threat has greatly attracted research attention in an attempt to come up with practical measures that will help to solve the problem at hand. Despite all the efforts that have been put in place to reduce antibiotic resistance burden, some gaps have to be filled by getting more effective drugs out there or acquiring better detection or isolation techniques. Secondary metabolites from natural sources have been shown to be promising leads in the pharmaceutical industry, but these sources have not been fully exploited.

1.3 Objectives

1.3.1 General Objective

To investigate the antimicrobial activity of fungal endophytes from selected medicinal plants.

1.3.2 Specific Objectives

1. To identify and characterize fungal endophytes from selected medicinal plants using molecular characteristics.
2. To screen fungal endophytes from medicinal plants for antimicrobial activities against selected human pathogens.
3. To characterize the antimicrobial secondary metabolites in selected medicinal plants' extracts.

1.4 Hypotheses

1. Fungal endophytes from medicinal plants have similar molecular characteristics.
2. Fungal endophytes are inactive against human pathogens.
3. Metabolites from medicinal plant extracts have similar physical and chemical characteristics.

1.5 Justification

Antibiotic resistance among disease-causing pathogens has resulted in emergence of multi-drug resistant pathogens and the increasingly immune-suppressed population now poses a new global health challenge. Despite research efforts made to achieve better cure, antibiotic resistance remains a global challenge. One promising, alternative, is the application of natural products in drug discovery, because it has the potential to provide a renewable, reproducible source of bioactive compounds. Medicinal plants are known to harbor endophytic microorganisms, which produce bioactive secondary metabolites. Medicinal plants produce phytochemicals of pharmaceutical significance. Therefore, there is a need to explore and obtain natural products from fungal endophytes inhabiting medicinal plants, with an intention of overcoming drug resistance menace.

References

Andremont, A. (2001). The future control of bacterial resistance to antimicrobial agents. *American Journal of Infection Control*, **29**: 256-258.

Calixto, B.J. (2000). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Brazilian Journal of Medical and Biological Research*, **33**: 179-189.

Charfi, F., Smaoui, H., & Kechrid, A. (2012). Non-susceptibility trends and serotype coverage by conjugate pneumococcal vaccines in a Tunisian pediatric population: A 10-year study. *Vaccine*, **30**:18-24.

Hoban, D., Baquero, F., Reed, V., & Felmingham, D. (2005). Demographic analysis of antimicrobial resistance among *Streptococcus pneumoniae*: worldwide results from PROTEKT 1999-2000. *International Journal of Infectious Diseases,* **9**:262-273.

Jenkins, S. G., Farrell, D. J., Patel, M., & Lavin, B. S. (2005). Trends in anti-bacterial resistance among *Streptococcus pneumoniae* isolated in the USA, 2000–2003: PROTEKT US years 1–3. *Journal of Infection*, **51**: 355-363.

Johnson, D. M., Stilwell, M. G., Fritsche, T. R., & Jones, R. N. (2006). Emergence of multidrug-resistant *Streptococcus pneumoniae*: report from the SENTRY Antimicrobial Surveillance Program (1999–2003). *Diagnostic Microbiology and Infectious Disease*, **56**: 69-74.

Lu, Y., Zhao, Y. P., Wang, Z. C., Chen, S. Y., & Fu, C. X. (2007). Composition and antimicrobial activity of the essential oil of *Actinidia macrosperma* from China. *Natural Product Research*, **21**: 227-233.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition*, **79**: 727-747.

Mbwambo, Z. H., Moshi, M. J., Masimba, P. J., Kapingu, M. C., & Nondo, R. S. (2007). Antimicrobial activity and brine shrimp toxicity of extracts of *Terminalia brownii* roots and stem. *BMC Complementary and Alternative Medicine*, **7**: 1-5.

N'guessan, J.D., Dinzedi, M.R., Guessennd, N., Coulibaly, A., Dosso, M., Djaman, A.J & Guede-Guina, F (2007). Antibacterial Activity of the Aqueous Extract of *Thonningia sanguine* Against Extended-Spectrum- β – Lactamases (ESBL) Producing *Escherichia coli* and *Klebsiella pneumoniae* Strains. *Tropical Journal of Pharmaceutical Research*, **6**: 779-783.

Phillipson, J.D (1994). Natural products as drugs. *Transactions of the Royal Society*. *Tropical Medicine Hygiene*, **88**: 17–19.

Song, J. H., Chang, H. H., Suh, J. Y., Ko, K. S., Jung, S. I., Oh, W. S., & Aswapokee, N. (2004). Macrolide resistance and genotypic characterization of *Streptococcus pneumoniae* in Asian countries: a study of the Asian Network for Surveillance of Resistant Pathogens (ANSORP). *Journal of Antimicrobial Chemotherapy*, **53**: 457-463.

Stone, J. K., Bacon, C. W., & White, J. F. (2000). An overview of endophytic microbes: endophytism defined. *Microbial Endophytes*, **3**: 29-33.

Strobel, G., Daisy, B., Castillo, U., & Harper, J. (2004). Natural products from endophytic microorganisms. *Journal of Natural products*, **67**: 257-268.

Tan, R. X., & Zou, W. X. (2001). Endophytes: a rich source of functional metabolites. *Natural Product Reports*, **18**: 448-459.

Varon, E. (2012). Epidemiology of *Streptococcus pneumoniae*. *Journal of Medicine and Infectious Diseases*, **42**: 361-365.

Weber, R. W., Kappe, R., Paululat, T., Mösker, E., & Anke, H. (2007). Anti-Candida metabolites from endophytic fungi. *Phytochemistry*, **68**: 886-892.

World Health Organization. (2014, April). WHO's first global report on antibiotic resistance reveals serious, worldwide threat to public health. In *Antimicrobial resistance–global surveillance report. Virtual Press Conference* (Vol. 30).

Yang, F., Xu, X. G., Yang, M. J., Zhang, Y. Y., Klugman, K. P., & McGee, L. (2008). Antimicrobial susceptibility and molecular epidemiology of *Streptococcus pneumoniae* isolated from Shanghai, China. *International Journal of Antimicrobial Agents*, **32**: 386-391.

Zhou, L., Yu, S.J., Gao, W., Yao, K.H., Shen, A. & Yang, Y (2011). Serotype distribution and antibiotic resistance of 140 pneumococcal isolates from pediatric patients with upper respiratory infections in Beijing, 2010.*Vaccine*, **29**: 7704-7710.

CHAPTER TWO

LITERATURE REVIEW

2.1 Medicinal plants

Plants have been considered one of the richest sources of raw materials for traditional as well as modern medicine, particularly in Africa and Asia (Miemanang *et al*., 2006). Africa contains a rich biodiversity of plant resources with a potential for drug discovery, out of which only a few species are used medicinally (Manach, 2004). The continent is located in the tropical and sub-tropical regions; where medicinal plants are most abundant. Plants in these areas accumulate unique secondary metabolites through evolution as a mode of surviving in a hostile environment (Calixto, 2000; Manach, 2004). Traditional medicine is the primary source of therapeutic systems in Africa. It is used by many people in the developing world and therefore plays a very significant role in the treatment of a variety of diseases (Phillipson, 1994). From surveys that were carried out in developed countries such as Germany and Canada, statistics indicated that at least 70% of the total population use the traditional medicine at least once (a day or a lifetime) (Gurib-Fakim, 2006). In this study, three medicinal plants were selected for phytochemical screening based on the available ethno botanical information concerning their medicinal uses in the traditional therapeutic system.

2.1.1 Genus *Warburgia*

The genus *Warburgia* belongs to the kingdom: Plantae, Division: Magnoliophyta, Class: Magnoliopsida, Order: Canellales, Family: Canellaceae. It is named after, Dr. Otto Warburg (1859-1938), a lecturer in Botany at the University of Berlin. The genus is one of four belonging to the tropical Canellaceae, a family that comprises of nine species (Esterhuyse, 1996), all of which are producers of aromatic oils. There are three species of *Warburgia*: *W. salutaris*, which is mainly found in southern Africa, along the coastal and submontane forest in Zimbabwe, Mozambique, Swaziland, and South Africa, where it is found in KwaZulu-natal, Mpumalanga, and Northern Province (Hollmann, Hertog, & Katan, 1996). *Warburgia ugandensis* Sprague and *W. stuhlmannii* Engl. are mainly found in eastern-central Africa. These species are known to be rich in sesquiterpenes of the drimane and colorotane skeletons (Kioy *et al*., 1990; Masimbye *et al*., 1999). They have been shown to possess antimicrobial, molluscidal, anticancer (Kubo *et al*., 1983) and antifungal properties (Kubo & Taniguchi, 1988).

2.1.1.1 *Warburgia ugandensis*

Warburgia ugandensis Sprague is a highly valued species within the traditional health systems in Eastern Africa (Plate 1). It has different names depending on the region where it grows including East African green wood, pepper-bark tree (English), Kenya greenheart, Muwiya (Luganda) among others. It is widely distributed in the tropical rain forest growing at altitudes between 1000m to 2000m (Maundu & Tengas, 2005). It is an evergreen tree with a height ranging from 4.5-30 m, 70 cm in diameter. The bark can be smooth or scaly, pale green or brown, and slash pink. The leaves are alternate, simple, dotted with glands, stipules absent. Petiole 1-5 mm long; blade oblong-lanceolate, elliptic or oblong-elliptical, 3-15 × 1.4-5 cm, tapering apex and base, margins entire, glossy dark green above, pale green and dull below. Midrib frequently slightly off- centre (Dale & Greenway, 1961).

Plate 1: *Warburgia ugandensis* leaves (source: www.iucn.org)

2.1.1.2 Research on *Warburgia ugandensis*

Research on this tree has mainly dwelt on the phytochemical compounds within the plant tissues. Extracts from leaves, bark and roots of this tree species have been subjected to numerous antimicrobial assays, and a further analysis of specific compounds with antimicrobial activity has been done. Compounds found to have antimicrobial activity include, Muzigadial, ugandensidal, perenporin B, cinnamolide, warburgin, and warburgiadione (Brooks & Draffan, 1969). Others include, 7-coloratadien-11, 12-dial, 4(13), 7-coloratadien-12, 11-olide and 7ß-hydroxy-4(13), 8-

coloratadien-11, 12-olide (Brooks & Draffan, 1969).The tree species is of pharmaceutical value in both human and animal medicine; this is attributed to the presence of biologically active unique compounds (Jansen & de Groot, 2004). This species is used in traditional health care systems to treat several diseases, including coughs, malaria, toothache, common cold, constipation, and fever (Kokwaro, 2009).

In animal medicine, studies show that a cytotoxic sesquiterpene isolated from *W. ugandensis* and characterized as Muzigadial is used in the treatment of typanasomiasis (Olila *et al*., 2001) and other parasitic infections (Kioy *et al*., 1990). The tree also comprises of other sesquiterpenoids such as bemadienolide, cinnamide, drimenol, warburgiadone, warburgin, ugandensidial and ugandensolide (Brooks & Draffan, 1969). Most of these possess broad antimicrobial activity as well as insect antifeedant activity.

2.1.2 Genus *Trichilia*

The genus *Trichilia* belongs to the Kingdom: Plantae, Subkingdom: Tracheobionta, Divison: Angiosperma, Class: Magnoliopsida, Subclass: Magnoliidae, Order: Rutales, Family: Meliaceae and Subfamily: Melioidae. The genus *Trichilia* is comprised of over 230 species and is mainly distributed in the tropical regions of America and Africa. Phytochemical studies have revealed that plants from this genus produce some phytochemicals including, terpenoids, flavonoids, limonoids, and steroids, which are of high pharmacological value (Rodrigues *et al*., 2010).

2.1.2.1 *Trichilia emetica* vahl meliaceae

The name '*Trichilia*' is of Greek origin meaning 'in 3 parts' referring to the 3-lobed fruit and 'emetica' means with emetic properties. The tree has different names including, Natal mahogany or woodland mahogany (English), ashapa (Yoruba), mtimai (Swahili) and mafura nut (trade name). *Trichilia emetica* is an evergreen tree (Plate 2) growing up to a height of about 21 cm, but occasionally some can grow to heights of 30 cm, the trunk is swollen at the base and sometimes becomes fluted with age. The bark is usually grey-brown or red-brown with fine, shallow striations and small scales (Orwa *et al*., 2009).

The branches erect or spread partly producing a pyramid-shaped crown when young. It is oval to round and dense when mature with a diameter exceeding 15 m. Leaves grow up to 50 cm, they are unevenly compound with 3-5 pairs of leaves including a terminal one, dark green and glossy on top; covered with short brownish hairs below, margins entire, veins prominent on the

lower surface. Flowers are creamy to pale yellow-green, produced on short, congested auxiliary panicles, with five thick petals. The fruits are rounded, furry, red-brown capsules to 3 cm across, split into 3-4 parts with the length of 14-18 mm black seeds, each with a fleshy scarlet or orange-red aril almost covering the seed (Orwa *et al*., 2009).

Plate 2: *Trichilia emetica* vahl *meliaceae* (Source: www.zimbabweflora.co.zw)

2.1.2.2 Research on *Trichilia emetica* vahl meliaceae

Due to its extensive biological properties, *T. emetica* has drawn the attention of scientists, and it has been widely investigated. Ethanolic extracts of the plant have been demonstrated to be a prostaglandin inhibitor hence showing its anti-inflammatory activity (McGaw *et al*., 2000). Previous studies have revealed that the methanolic extracts exhibit good antiplasmodial activity (El Tahir *et al*., 1999). Studies by Spargand *et al.* (2000) demonstrated anti-schistosomiasis activity while methylene chloride extracts of *T. emetica* leaves exhibited good *in vitro* antitrypanosomal activity against *Trypanosoma brucei* (Hoet *et al*., 2004). Aqueous leaves extract of the same demonstrated analgesic activity (Sanogo *et al*., 2006).

Studies done on *T. emetica* have established that the plant contains many phytochemicals of biological significance especially for the treatment of many diseases. Such compounds include sesquiterpenes, triterpenes with A-seco-Ring and limonoids, which include the trichilins (Traore *et al*., 2007). *Trichilia emetica* is used to treat diseases such as malaria, epilepsy, mental illness, typhoid fever, abdominal pains dermatitis and hemorrhoids (Komane *et al*., 2011). It is also used in the treatment of abscesses, gonorrhea, and eczema (Germano *et al*., 2005). The main parts that are often used are the leaves and roots. Traditionally, these parts are administered in powder

forms, maceration, or the form of a decoction. Concoctions are usually administered orally, via a body bath or through the topical application on the skin (Tchacondo *et al*., 2012). From these reports, antibacterial and antifungal activity of the leaves extracts of *T. emetica* has not been extensively studied, thus, the aim of this research was to screen different fractions of the leaves extracts for phytochemicals present.

2.1.3 Genus *Albizia*

The genus *Albizia* is classified as follows, Kingdom; Plantae, Order; Fabales, Family; Fabaceae, Subfamily; Mimosoideae and Tribe; Ingae (Karuppannan *et al*., 2013). The genus comprises of approximately 150 species that are widely distributed in the tropical regions of Africa and central South America. From previous phytochemical studies done on this genus, it has been demonstrated that the plants in this genus produce many secondary metabolites that have medicinal value. Such compounds are saponins, terpenes, alkaloids, and flavonoids (Singab & Bahgat, 2015).

2.1.3.1 *Albizia gummifera*

Albizia gummifera is a deciduous tree, which is approximately 4.5-30m high with branches ascending to a flat top. The leaves are bipinnate in 5-7 pairs; leaflets are dark green and almost similar in size, obliquely rhombic to sub falcate, apex obtuse or acute, 10-25 by 4-12 mm (Plate 3). The tree is widely distributed in the rainforests, riverine forests, and lowland areas (Orwa *et al*., 2009).

Plate 3: *Albizia gummifera* (Source: www.zimbabweflora.co.zw)

2.1.3.2 Research on *Albizia gummifera*

Phytochemical studies done on plants in the genus Albizia show that they contain different bioactive secondary metabolites. Triterpenic saponins were found in *A. gummifera* (Cao *et al*., 2007). Spermine alkaloids were found in *A. gummifera* (Rukunga *et al*., 2007). Six triterpenoid glycosides were isolated from *A. gummifera* including monodesmosidicsaponin, bidesmosidicsaponin, stigmasterol, two new sapogenin glycosides containing macrinic acid and gamma lactone and a sapogenin lactone (Zheng *et al*., 2006). Traditionally, *A. gummifera* has been used in the treatment of ailments. For instance, the root decoction of this medicinal plant has been used in the treatment of malaria, stomach pains, skin infections, scabies, and psychiatric problems (Kokwaro, 2015). Previous studies on *A. gummifera* have demonstrated that lipophilic extracts of the same have anti-trypanosomal activity as well as antibacterial activity (Orwa *et al*., 2009).

2.2 Endophytes

Endophytes are bacteria or fungi that live in healthy tissues of plants intercellularly or intracellularly without causing any disease symptom (Wilson, 1995). It is presumed that they protect the plant against pathogenic infections by producing antimicrobial compounds such as secondary metabolites and in return, the host plant provides these endophytes with nutrients (Yu *et al*., 2010). The relationship between endophytes and their hosts may range from latent phytopathogenesis to mutualistic symbiosis (Sturz *et al*., 1997). Currently, endophytes are viewed as a rich source of bioactive compounds (Cragg & Newman, 2013). They produce secondary metabolites that are active against several disease-causing pathogens and thus form a good source of potential novel drugs (Strobel, 2002).

Endophytes play various important roles in the host plant such as increasing plant biomass; improve plant resistance to pathogen damage as well as improving plant adaptation to stress (Arnold *et al*., 2003). Endophytes are ubiquitous in nature (Gonthier, 2006). Strobel *et al*. (2004) reported that plants growing in areas of vast biodiversity have the potential of housing diverse endophytes. For instance, the tropical rainforest forms a resourceful habitat for endophytes. Other important ecological factors that affect endophyte diversity include plants growing in special habitats. Kumaresan & Suryanarayanan (2001) isolated a number of endophytes from the mangrove forest. Ji *et al*. (2004) isolated an endophytic fungus from a

traditional medicinal plant. Plants occupying ancient landmasses have been found to harbor a lot more endophytes than other plants; this was clearly studied by Strobel & Daisy (2003). In addition, plants that are usually surrounded by pathogen-infected plants tend to have quite a lot of endophytes.

2.2.1 Endophytic Fungi

For a long time, the definition of endophytic fungi has been changing but Clay (1990) who defined endophytic fungi as asymptomatic fungi that are usually found in aerial plant tissues gave the best description. Endophytic fungi play a role in increasing resistance in plants to abiotic stress, herbivores and pathogens (Saikkonen *et al*., 1998). Medicinal plants form the richest reservoir of novel bioactive metabolites (Sappapan *et al*., 2008). Research has demonstrated that endophytic fungi inhabiting medicinal plants play a significant role in the production of secondary metabolites, which have antibacterial, antifungal, antioxidant, and anticancer activities (Arnold *et al*., 2003). A number of medicinal plants have been screened for endophytic fungi (Stierle *et al*., 1993). Compounds produced by these endophytic fungi possess unusual chemical structures with particular bioactivities and thus this field poses an interesting area for exploitation of novel compounds of pharmaceutical importance. Fungal endophyte diversity is highest in tropical forests where woody angiosperm diversity is also higher (Banerjee, 2011). Studies on endophytic fungi of medicinal plants have been given much attention, as they tend to produce natural products beneficial to man.

2.2.2 Techniques For Isolation Of Endophytic Fungi

Endophytic fungi isolation methods differ depending on the method used for surface sterilization of the host plant tissue (leaves, stems, roots, barks, flowers, fruits, and seeds) and the choice of culture media. The disinfection process can have an effect on the detection of endophytic fungi; usually the plant surface is sterilized with a strong oxidant or a disinfectant agent for a specific period. Commonly used oxidants or detergents include 3% hydrogen peroxide, 2-10% sodium hypochlorite or 70-95% ethanol (Larran *et al*., 2007). Commonly used culture media include Potato Dextrose Agar (PDA), malt extract agar, Saboraud dextrose agar (SDA) and yeast malt agar; supplemented with standard antibiotics such as streptomycin, chloramphenicol, penicillin, ampicillin, among others to prevent growth of contaminating bacteria. Incubation temperature for endophytic fungi growth varies between 25-28ºC (Larran *et al*., 2007).

2.2.3 Metabolites of Pharmaceutical Value From Endophytic Fungi

In 1993, the discovery of the multi-billion anticancer compound paclitaxel (taxol®) from the endophytic fungi *Taxomyces andreanae* which inhabits the plant *Taxus brevifolia* (Stierle *et al*., 1993) marked an important successful step in the field of drug discovery. Since then extensive research has been done on endophytic fungi from various plant sources in an attempt to isolate and identify novel bioactive compounds of pharmaceutical value. As a result, a number of novel compounds with spectacular antimicrobial, cytotoxic, and anticancer activities have been discovered. These compounds are mainly categorized as quinines, isocoumarins, lactones, terpenoids, phenylpropanoids, phenols, and alkaloids (Yu *et al*., 2010). Paclitaxel was found to be active against a number of tumors including, lung, head, breast, and ovarian tumors. Besides the endophyte *T. andreanae*, a number of other endophytes with different plant origins have been studied extensively and it has been demonstrated that they can also produce paclitaxel. These endophytic fungi include *Pestaliotiopsis microspora* (Strobel *et al*., 1996), *Periconia* sp. (Li *et al*., 1998), *Bartalinia robillardoides* and *Colletotrichum gloeosporioides* (Gangadevi & Muthumary, 2008). Podophyllotoxin, a precursor of several anti-cancer drugs, was recently discovered to be produced by the endophytic fungus *Fusarium oxysporum*, which occurs naturally as an endophyte in the medicinal plant *Juniperus recurva* (Kour *et al*., 2008).

In a study, the endophyte *Nodulisporium* sp. (Xylariaceae) isolated from the plant *Erica arborea* was found to produce a number of compounds with antifungal, antibacterial and algicidal activities (Dai *et al*., 2009). The endophytic fungus *Phomopsis* sp. isolated from the leaves of *Laurus azorica* produced two new metabolites, cycloepoxytriol B and cycloepoxylactones which were shown to have potential antibacterial and antifungal activities (Hussain *et al*., 2009).

2.3 Characterization Of Endophytic Fungi

2.3.1 Morphological Identification

Conventional fungi taxonomy is based on comparative morphology and on the development of sexual reproductive structures. Endophytic fungi identification relies on the taxonomic expertise of a mycologist. Generally, fungal endophytes with similar culture characteristics are grouped together into one group called the morphospecies, which represents the functional taxonomic unit for endophytic species (Arnold *et al*., 2000). However, these

conventional methods for detection and identification are laborious as well as time consuming and non-sporulating fungi are difficult to identify. These characteristics are mainly observed in endophytic fungi of the class ascomycetes, basidiomycetes and some dematiaceous. Therefore, molecular tools come in handy in terms of identification of endophytic fungi. Microscopic identification of endophytic fungi can be done depending on whether the fungus produces reproductive structures while growing on culture media. In this case, slides are prepared from the cultures using lactophenol cotton blue stain and examined with a bright field and phase contrast microscope.

2.3.2 Molecular Identification

The use of molecular tools in the identification of endophytic fungi and other microorganisms is a powerful and sensitive technique that is used to classify non-sporulating endophytic genera and microbial strains in diverse hierarchical taxonomic levels. Endophytic fungi are identified based on the sequence of the Internal Transcribed Spacer (ITS) region of the larger subunit of the rDNA gene. The information contained in this region has been extensively used in characterization studies identification and detection typing and for the establishment of phylogenetic relationships (Smit *et al*., 1999; Sutar *et al*., 2004; Wu *et al*., 2003; LoBuglio & Taylor, 2002). After sequencing of the ITS region, the generated sequences are compared with sequences of other taxa deposited in public databases.

2.4 Fermentation Techniques And Crude Extract Production

In the microbial growth phase, secondary metabolite production begins at the stationary phase; these are usually compounds with unique chemical structures. Their production begins when a vital source of nutrient such as carbon, nitrogen or phosphate are depleted (Barrios-González & Mejia, 1996). Fungal endophytes should be obtained in pure culture and optimal media. Growth conditions must be determined before fermentation begins, as this will determine crude extract production. Factors that quantitatively and qualitatively affect production of secondary metabolites include degree of aeration, temperature, media composition, pH and culture duration (Barrios-González & Mejia, 1996).

Different types of media can be used to cultivate fungi, these are; Potato Dextrose Agar (PDA), Yeast Malt Agar (YMA), Oatmeal Agar (OMA), Corn Meal Agar (CMA) among others. The type of media is dependent on the purpose and type of species (Hölker *et al*., 2004).

Fermentation techniques used in the cultivation of fungal endophytes include submerged fermentation (SmF) or liquid fermentation and solid-state fermentation (SSF) (Rosa *et al*., 2011). Although the two techniques are different in terms of operation both can be used to identify secondary metabolites produced by fungal endophyte. The appropriateness of a given procedure is usually evaluated based on the objective of the study and available resources.

2.5 Infectious Diseases and Antibiotic Resistance

Infectious diseases account for almost one quarter of all deaths worldwide. Since the introduction of penicillin in the 1940s, antibiotics have played a big role in the treatment of bacterial infections. Although there have been significant advances in research, antibiotic resistance by disease causing pathogens in the effective first-line drugs still remain to be a global public health challenge (Davies *et al*., 2013). For most of sub-Saharan Africa, antibiotics are already very limited and any breach on the list leads to nearly total loss of treatment choices for many severe infections.

Antibiotic resistance is observed in a number of bacteria that cause bloodstream infections (sepsis), diarrhea, pneumonia, urinary tract infections, respiratory infections and gonorrhoea. For instance, the probability of death among people with methicillin-resistant *Staphylococcus aureus* (MRSA) is quite high compared to populations with a non-resistant form of the same (WHO Report, 2014). Resistance also increases the cost of health care with lengthier stays in hospital and more intensive care required. With such problems on the rise, there is the need to find efficient and affordable solutions in an attempt to come up with new lines of pharmaceuticals from untapped natural product resources.

References

Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D., & Kursar, T. A. (2000). Are tropical fungal endophytes hyperdiverse? *Ecology letters*, **3**: 267-274.

Arnold, A. E., Mejía, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N., & Herre, E. A. (2003). Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences*, **100**: 15649-15654.

Banerjee, D. (2011). Endophytic fungal diversity in tropical and subtropical plants. *Research Journal of Microbiology*, **6**: 54-62.

Barrios-González, J., & Mejia, A. (1996). Production of secondary metabolites by solid state fermentation. *Biotechnology Annual Review*, **2**: 83-121.

Brooks, C. J & Draffan, G. H. (1969). Sesquiterpenoids of *Warburgia ugandensis* Ugandensolide and Ugandensidial. *Tetrahedron*, **25**: 2887 -2898.

Cao, S., Norris, A., Miller, J. S., Ratovoson, F., Razafitsalama, J., Andriantsiferana, R., & Kingston, D. G. (2007). Cytotoxic Triterpenoid Saponins of *Albizia gummifera* from the Madagascar Rain Forest. *Journal of Natural Products*, **70**:361-366.

Clay, K. (1990). Fungal endophytes of grasses. *Annual Review of Ecology and Systematics*, **21**: 275-297.

Cragg, G. M., & Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochimica et Biophysica Acta (BBA)-General Subjects*, **1830**: 3670-3695.

Dai, J., Krohn, K., Draeger, S., & Schulz, B. (2009). New Naphthalene-Chroman Coupling Products from the Endophytic Fungus, *Nodulisporium* sp. from *Erica arborea*. *European Journal of Organic Chemistry*, **10:** 1564-1569.

Dale, I. R., & Greenway, P. J. (1961). *Kenya Trees and Shrubs* (No. 04; QK491. K4, D3.).

Davies, S. C., Fowler, T., Watson, J., Livermore, D. M., & Walker, D. (2013). Annual Report of the Chief Medical Officer: infection and the rise of antimicrobial resistance. *The Lancet*, **381**: 1606-1609.

El Tahir, A., Satti, G. M., & Khalid, S. A. (1999). Antiplasmodial activity of selected Sudanese medicinal plants with emphasis on *Maytenus senegalensis* (Lam.) Exell. *Journal of Ethnopharmacology*, **64**: 227-233.

Esterhuyse, C. J. (1996). Trees of the year. Issued by the forestry chief directorate of the department of water affairs and forestry, Pretoria, SouthAfrica.

Gangadevi, V., & Muthumary, J. (2008). Isolation of *Colletotrichum gloeosporioides*, a novel endophytic taxol-producing fungus from the leaves of a medicinal plant, *Justicia gendarussa*. *Mycologia balcanica*, **5**: 1-4.

Gonthier, P., Gennaro, M., & Nicolotti, G. (2006). Effects of water stress on the endophytic mycota of *Quercus robur*. *Fungal Diversity*, **21**: 69-80.

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*, **27**: 1-93.

Hoet, S., Opperdoes, F., Brun, R., Adjakidjé, V., & Quetin-Leclercq, J. (2004). *In vitro* antitrypanosomal activity of ethno pharmacologically selected Beninese plants. *Journal of Ethnopharmacology*, **91**: 37-42.

Hölker, U., Höfer, M., & Lenz, J. (2004). Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Applied Microbiology and Biotechnology*, **64**: 175-186.

Hollman, P. C., Hertog, M. G., & Katan, M. B. (1996). Role of dietary flavonoids in protection against cancer and coronary heart disease. *Biochemical Society Transactions*, **24**: 785-789.

Hussain, H., Akhtar, N., Draeger, S., Schulz, B., Pescitelli, G., Salvadori, P., & Krohn, K. (2009). New Bioactive 2, 3-Epoxycyclohexenes and Isocoumarins from the endophytic fungus *Phomopsis* sp. from *Laurus Azorica*. *European Journal of Organic Chemistry,* **5**: 749-756.

Jansen, B. J., & de Groot. A. (2004). Occurrence, biological activity and synthesis of drimane sesquiterpenoids. *Natural Product Reports*, **21**: 449–477.

Ji, Z., Wu, W., Wang, M., & Gu, A. (2004). Identification of fungicidal compounds from endophytic fungi *Fusarium proliferatum* in *Celastrus angulatus*. *Journal of Northwest Sci-Tech University of Agriculture and Forestry (Natural Science Edition)*, **33**: 61-64.

Karuppannan K., Priyadharshini, S. D., & Sujatha, V. (2013). Phyto pharmacological properties of *Albizia* species: A review. *International Journal of Pharmaceutical Science*, **5**: 70-73.

Kioy, D., Gray A. L., & Waterman, P. G. (1990). A comparative study of the stem bark drimane sesquiterpenes and leaf volatile oils of *Warburgia ugandensis* and *Warburgia stuhlmanii*. *Phytochemistry*, **29**: 3535 – 3538.

Kokwaro, J. O. (2015). *Classification of east African crops*. University of Nairobi Press.

Kokwaro, J.O. (2009). *Medicinial Plants of East Africa.* Nairobi:University of Nairobi Press.

Komane, B. M., Olivier, E. I., & Viljoen, A. M. (2011). *Trichilia emetica* (Meliaceae)–A review of traditional uses, biological activities and Phytochemistry. *Phytochemistry Letters*, **4**: 1-9.

Kour, A., Shawl, A. S., Rehman, S., Sultan, P., Qazi, P. H., Suden, P., & Verma, V. (2008). Isolation and identification of an endophytic strain of *Fusarium oxysporum* producing podophyllotoxin from *Juniperus recurva*. *World Journal of Microbiology and Biotechnology*, **24**: 1115-1121.

Kubo, I., & Taniguchi, M. (1988). Polygodial an antifungal potentiator. *Journal of Natural Products,* **51**: 22-29.

Kubo, I., Matsumoto, T., Kakooko, A.B., & Mubiru, N.K., (1983). Structure of mukaadial, a molluscicidal from *Warburgia* plant. *Chemistry Letters*, **7**: 979–980.

Kumaresan, V., & Suryanarayanan, T.S. (2001). Occurrence and distribution of endophytic fungi in a mangrove community. *Mycological Research*, **105**:1388–91.

Larran, S., Perelló, A., Simón, M. R., & Moreno, V. (2007). The endophytic fungi from wheat (*Triticum aestivum* L.). *World Journal of Microbiology and Biotechnology*, **23**: 565-572.

Li, J. Y., Sidhu, R. S., Ford, E. J., Long, D. M., Hess, W. M., & Strobel, G. A. (1998). The induction of taxol production in the endophytic fungus *Periconia* sp. from *Torreya grandifolia*. *Journal of Industrial Microbiology and Biotechnology*, **20**: 259-264.

Mashimbye, M. J., Maumela, M. C., & Drewes, S. E. (1999). A drimane sesquiterpinoid lactone from *Warburgia salutaris*. *Phytochemistry*, **51**: 435- 438.

Maundu, P., & Tengnas, B. (2005). Useful Trees and Shrubs for Kenya. World Agroforestry Centre, Eastern and Central Africa Regional Programme. Nairobi, Kenya.

McGaw L.J., Jager A.K., & van Staden J. (2000). Antibacterial, antihelminthic and antiamoebic activity in South African medicinal plants, *Journal of Ethnopharmacology*, **72**: 247–263

Miemanang, R. S., Krohn, K., Hussain, H., & Dongo, E. (2006). Paullinoside a and paullinomide a: a new cerebroside and a new ceramide from leaves of *Paullinia pinnata*. *Journal of Chemical Sciences*, **61**: 1123-1127.

Olila, D. B., Olwa, O., & Opuda, J. (2001). Antibacterial and antifungal activities of extracts of *Zanthoxylum chalybeum* and *Warburgia ugandensis*, Ugandan medicinal plants. *African Health Sciences*, **1**: 66-72.

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R., & Simons, A. (2009). Agroforestry Database: a tree reference and selection guide version 4.0. *World Agroforestry Centre, Kenya*, *15*.

Rodrigues, V. F., Carmo, H. M., Braz, F. R., Mathias, L., & Vieira, I. J. (2010). Two new terpenoids from *Trichilia quadrijuga* (Meliaceae). *Natural Product Communications*, **5**: 179-184.

Rosa, L. H., Cota, B. B., Rosa, C. A., Zani, C. L., Vieira, M. L., Johann, S., & Alves, T. M. (2011). Endophytic fungi of tropical forests: a promising source of bioactive prototype molecules for the treatment of neglected diseases (pp. 1-18). INTECH Open Access Publisher.

Rukunga, G. M., Muregi, F. W., Tolo, F. M., Omar, S. A., Mwitari, P., Muthaura, C. N., & Iraqi, F. W. (2007). The antiplasmodial activity of spermine alkaloids isolated from *Albizia gummifera. Fitoterapia*, **78**: 455-459.

Saikkonen, K., Faeth, S. H., Helander, M., & Sullivan, T. J. (1998). Fungal endophytes: a continuum of interactions with host plants. *Annual Review of Ecology and Systematics*, **29**: 319-343.

Sanogo, R., Maiga, A., & Diallo, D. (2006). Analgesic and anti-inflammatory activities of *Maytenus senegalensis*, *Stereospermum kuntrianum* and *Trichilia emetica* used in traditional treatment of dysmenorrheal in Mali. *Journal of pharmaceutical and African Traditional Medicinal Plants*, **14**: 123-36.

Sappapan, R., Sommit, D., Ngamrojanavanich, N., Pengpreecha, S., Wiyakrutta, S., Sriubolmas, N., & Pudhom, K. (2008). 11-Hydroxymonocerin from the plant endophytic fungus *Exserohilum rostratum*. *Journal of Natural Products*, **71**: 1657-1659.

Singab, A. N., & Bahgat, D. (2015). Saponins from Genus *Albizia*: Phytochemical and Biological Review. *Medicinal & Aromatic Plants*, **3**: 2147-0142.

Smit, E., Leeflang, P., Glandorf, B., van Elsas, J. D., & Wernars, K. (1999). Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Applied and Environmental Microbiology*, **65**: 2614-2621.

Spargand, S. G., Van Staden, J., & Jäger, A. K. (2000). Efficiency of traditionally used South African plants against schistosomiasis. *Journal of Ethnopharmacology*, **73**: 209-214.

Stierle, A., Strobel, G., & Stierle, D. (1993). Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science*, **260**: 214-216.

Strobel, G. A. (2002). Rainforest endophytes and bioactive products. *Critical Reviews in Biotechnology*, **22**: 315-333.

Strobel, G., & Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews*, **67**: 491-502.

Strobel, G., Yang, X., Sears, J., Kramer, R., Sidhu, R. S., & Hess, W. M. (1996). Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. *Microbiology*, **142**: 435-440.

Sturz, A. V., Christie, B. R., Matheson, B. G., & Nowak, J. (1997). Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils*, **25**: 13-19.

Sutar, R., David, J. K., Ganesan, K., Ghosh, A. K., Singhi, S., Chakrabarti, A., & Bachhawat, A. K. (2004). Comparison of ITS and IGS1 regions for strain typing of clinical and non-clinical isolates of *Pichia anomala*. *Journal of Medical Microbiology*, **53**: 119-123.

Tchacondo, T., Karou, S. D., Agban, A., Bako, M., Batawila, K., Bawa, M. L., & de Souza, C. (2012). Medicinal plants use in central Togo (Africa) with an emphasis on the timing. *Pharmacognosy Research*, **4**: 92-103.

Traore, M., Zhai, L., Chen, M., Olsen, C. E., Odile, N., Pierre, G. I., & Christensen, S. B. (2007). Cytotoxic kurubasch aldehyde from *Trichilia emetica*. *Natural Product Research*, **21**: 13-17.

Wilson, D. (1995). Endophyte: the evolution of a term, and clarification of its use and definition. *Oikos*, **73**: 274–276.

Wu, Z., Tsumura, Y., Blomquist, G., & Wang, X. R. (2003). 18S rRNA gene variation among common airborne fungi and development of specific oligonucleotide probes for the detection of fungal isolates. *Applied and Environmental Microbiology*, **69**: 5389-5397.

Wube, A. A., Bucar, F., Gibbons, S., & Asres, K. (2005). Sesquiterpenes from *Warburgia ugandensis* and their antimycobacterial activity. *Phytochemistry*, **66**:2309-2315.

Yu, H., Zhang, L., Li, L., Zheng, C., Guo, L., Li, W., & Qin, L. (2010). Recent developments and future prospects of antimicrobial metabolites produced by endophytes. *Microbiological Research*, **165**: 437-449.

Zhang, H. W., Song, Y. C., & Tan, R. X. (2006). Biology and chemistry of endophytes. *Natural Product Reports*, **23**: 753-771.

Zhou, L., Zhao, J., Xu, L., Huang, Y., Ma, Z., Wang, J., & Jiang, W. (2009). Antimicrobial compounds produced by plant endophytic fungi. *Fungicides: Chemistry, Environmental Impact and Health Effects*, **91**:116-119.

CHAPTER THREE

MOLECULAR CHARACTERIZATION OF FUNGAL ENDOPHYTES FROM KENYAN MEDICINAL PLANTS

3.1 Abstract

Traditional methods of fungal identification (morphological identification) have proven to be a challenge in the identification of non-sporulating fungal endophytes. The advent of molecular identification techniques has offered an effective method for identification of non-sporulating fungal endophytes to species level. Non-sporulating and sporulating fungal endophytes from leaves of 22 medicinal plants growing in the Kakamega tropical rainforest were characterized using molecular tools. This was achieved by sequencing the amplified ITS1-5.8S-ITS2 region of the fungal genome using forward ITS1F (CTTGGTCATTTAGAGGAAGTAA) and reverse ITS4 (TCCTCCGCTTATTGATATGC) primers. Amplified PCR products were sequenced and phylogenetic evolutionary relationships were evaluated from the generated sequences using the Maximum Likelihood algorithm. Fifty-nine out of 65 endophyte isolates investigated were successfully identified to the genus level while the remaining eight could not be identified. The DNA sequences retrieved were shown to belong to three classes of fungal endophytes namely *Sordariomycetes*, *Eurotiomycetes,* and *Dothideomycetes* distributed in nine genera: *Fusarium, Colletotrichum, Trichothecium, Phomopsis*, *Pestalotiopsis*, *Cladosporium*, *Aspergillus*, *Phoma,* and *Chaetomium*. Results obtained from this study show that medicinal plants are a host to a great diversity of fungal endophytes.

3.2 Introduction

Fungal endophytes are microorganisms that live within host plant tissues without causing any disease symptom (Petrini *et al*., 1992). They have been considered to produce secondary metabolites of pharmaceutical value (Pillay & Zambon, 1998; Espinel *et al*., 2001). It is estimated that there are about more than a million fungal endophytes that colonize different plant species (Huang *et al*., 2007). However, there is little knowledge on the biology of these fungal endophytes thus their isolation and characterization is important. Conventional fungi taxonomy is based on comparative cultural and morphological characteristics. In this case, fungal endophytes with similar cultural characteristics are grouped together into one group (Arnold *et*

al., 2000). But these methods for detection and identification are laborious as well as time-consuming (Wu *et al*., 2003), and non-sporulating fungi are difficult to identify on a morphological basis. Therefore, molecular techniques come in handy with respect to identification of fungal endophytes. These methods have proven to be robust and sensitive to classify non-sporulating endophytic fungi. Fungal endophytes are identified based on the sequence of the ITS region of the larger subunit of the rDNA gene. The ITS regions are the non-coding sequences in the fungi genome, which gets transcribed along with the rDNA coding genes and are therefore seen in the precursor transcripts of rDNA (Peay, Kennedy, & Bruns, 2008). The ITS regions are usually highly conserved because of low evolutionary pressure acting on the non-functional sequences. For this reason, ITS markers have been used for elucidating relationships among closely related genera (Baldwin *et al*., 1995; Kim *et al*., 1996).

The most commonly used standard ITS primers are ITS1 and ITS4 (White *et al*., 1990). Besides, several taxon-specific primers have been described to allow for selective amplification of fungal sequences (Gardes & Bruns, 1993). Following the sequencing of ITS region, generated sequences are compared with other ITS sequences of organisms represented in public databases such as the GenBank database. The purpose of this study was to characterize taxonomically and establish the phylogenetic relationships of fungal endophytes isolated from leaves of selected Kenyan medicinal plants.

3.3 Materials and Methods

3.3.1 Endophyte Isolation

Sampling was done in the Kakamega tropical rainforest, which lies at 0º 10'- 0º 21' N, 34º 58' E. The leaf samples of selected medicinal plants were collected based on ethno botanical information provided and were place in sterile plastic bags and processed within a few hours after collection. Endophytic fungi were isolated from the leaves using a modification of the method described by Zinniel *et al*. (2002). In this method, the leaves of the selected plants were washed under running tap water to remove any soil or other foreign materials. Afterwards, they were surface sterilized for three minutes using 70% ethanol followed by soaking in 1% sodium hypochlorite for one minute and rinsed three times with sterile distilled water to remove any traces of the disinfectant. The surface sterilized leaves were cut aseptically into sections approximately $2cm^2$, which were then superficially cut repeatedly and inoculated in petri dishes

containing PDA (39 g/l), amended with streptomycin sulphate (250 mg/l). The inoculated plates were placed in an incubator at 25 ± 2ºC for 1- 4 weeks. The petri dishes were monitored after every three days to check for fungal growth. Pure cultures were then prepared by sub-culturing fungal mycelia of each endophyte isolate into sterile petri dishes containing PDA without antibiotics. The plates were incubated at 25 ± 2ºC for 1- 4 weeks and photographs of fully grown pure cultures taken.

3.3.2 DNA Extraction

DNA extraction of endophytic fungi was done using BIO BASIC EZ-10 Spin column miniprep kit following manufacturer's instructions (Bio Basic Inc.). Fungal endophyte mycelia were obtained from one-week-old cultures and 60 mg of the mycelia was placed into a 1.5 ml screw cap reaction tube. Approximately 5-8 of 1.4 mm precellys ceramic beads were added to the tube and the sample material was covered with 150 µl of plant cell lysis (PCL) buffer. The mixture was homogenized in a precellys 24 homogenizer using program 5 (6.0m/s for 2×40s) and then incubated on a heating block set at 65ºC for 20 minutes. After incubation, 25 µl of protein precipitation (PP) solution was added to the sample mix and incubated for 15 minutes on ice. The sample was centrifuged at 13 400g (12 000 revolutions per minute) at 4ºC for 2 minutes. The clear lysate formed because of centrifugation was transferred onto an EZ-10 Spin Column using a micropipette. Phosphate Buffer Saline (PBS) (300 µl) was added to the lysate and the samples were incubated for 3 minutes at room temperature with occasional mixing. They were centrifuged at 13 400g (12 000 rpm) at 4ºC for 30 seconds and thereafter the flow through was discarded. Wash solution (500 µl) was added to the sample and centrifuged at 13 400g (12 000 rpm) at room temperature for 30 seconds and the flow through was discarded. This step was repeated twice. Then the samples were centrifuged at 13 400g (12 000 rpm) at room temperature for 1 minute. The collection tubes of the EZ-10 Spin column were replaced with clean 1.5 ml reaction tubes. About 50 µl 65ºC warm elution buffer was added to the filtrate and incubated at room temperature for 2-3 minutes, after which a final centrifugation at 9000 g was done for 2 minutes. The EZ-10 Spin columns were discarded and the DNA was stored at 4ºC for further use. The remaining fungal endophyte cultures were stored at -20 ºC for future reference.

3.3.3 Polymerase Chain Reaction (PCR) Amplification

The Internal Transcribed Spacer (ITS) region of the ribosomal DNA (rDNA) was amplified by conducting a Polymerase Chain Reaction (PCR). Polymerase Chain Reaction amplification was done in a final volume of 25 µl by mixing 2 µl of the genomic DNA (2 ng) with 23 µl of the master mix. The latter was prepared by adding 0.5 µl of the forward primer ITS1F (CTTGGTCATTTAGAGGAAGTAA) into a 2ml eppendorf tube, followed by 0.5µl of the reverse primer ITS4 (TCCTCCGCTTATTGATATGC). About 12.5µL of the jump start ready mix (20 mM Tris-HCl, pH 8.3, 100 mM KCl, 4 mM $MgCl_2$, 0.002% gelatin, 0.4 mM dNTPs, inert dye, stabilizers, 0.03 unit/mL Taq DNA polymerase, JumpStart™ Taq Ready Mix™ and 9.5µl of distilled water) (White *et al*., 1990; Gardes & Bruns, 1993). The mixture was vortexed for 30 seconds. About 2 µl of distilled water mixed with 23 µl of master mix was used as the negative control. Amplification was done in a thermal cycler (Applied Biosystems™) with an initial denaturation of 5 minutes at 94 ºC, followed by 35 cycles of denaturation for 30 seconds at 94ºC, 1 minute for annealing at 52ºC, 1 minute for elongation at 72ºC and a final elongation of 10 minutes at 72ºC. The quality and quantity of PCR products (3µL) were checked by electrophoresis on a 0.8% agarose gel with midori green dye in 1 × TAE buffer for 30 minutes at 100 volts and visualization was done by use of UV transilluminator (Nippon Genetics Europe GMbH).

3.3.4 Sequencing and Phylogenetic Analyses

The amplified PCR products were purified according to BioBasic EZ-10 spin column PCR purification kit following manufacturer's instructions. The PCR reaction mixture was placed into 1.5ml microfuge tubes and 5ml of buffer B3 added. The mixture was then transferred to EZ-10 columns, left to stand for about 2 minutes at room temperature, and centrifuged at 8000g for 30 seconds. The flow through was removed and about 750µl of wash solution was added to the column and further centrifuged at 9000g for 30 seconds. Washing step was repeated twice. The columns were transferred into clean 1.5ml microfuge tubes and about 20µl of elution buffer added. This was incubated at room temperature for 2 minutes and then centrifuged at 9000g for 1 minute to elute the DNA. Sample preparation for sequencing was done by preparing 1:1 primer (ITS1F and ITS4): distilled water dilution in addition to the amplified DNA of fungal endophytes. Sequencing was done using the automated illumina genome analyzer IIX DNA sequencing machine. The sequences were then checked for sequencing errors using Geneious

version 9.0 software (Kearse *et al*. 2012). Furthermore, the cleaned sequences were used to retrieve closely related sequences for comparison using ITS sequence data from strains available in the public database GenBank (http://www.ncbi.nlm.nih.gov/genbank/). This was achieved by using Basic Local Alignment Search Tool (BLAST) N sequence match routines. However, the sequences were not deposited in the nucleotide sequence database. The sequences were then aligned using ClustalX software program version 2.1 (Larkin *et al*., 2007). Alignments obtained were used to calculate distance matrices and construct phylogenetic trees. Phylogenetic and molecular evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 (Tamura *et al*., 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). The tree with the highest log likelihood (-989.4346) is shown in Figure 2. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 47 nucleotide sequences; codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 181 positions in the final data set. Evolutionary analyses were conducted in MEGA version 6 (Tamura *et al*., 2013).

3.4 Results

3.4.1 Isolation of Fungal Endophytes

Fifty-five endophytic fungal isolates were successfully obtained from surface sterilized fresh leaves of 23 indigenous medicinal plants sampled in the Kakamega tropical rainforest (Table 1). Pure cultures (Plate 4) of fungal endophytes were obtained from subsequent sub culturing of the isolated strains.

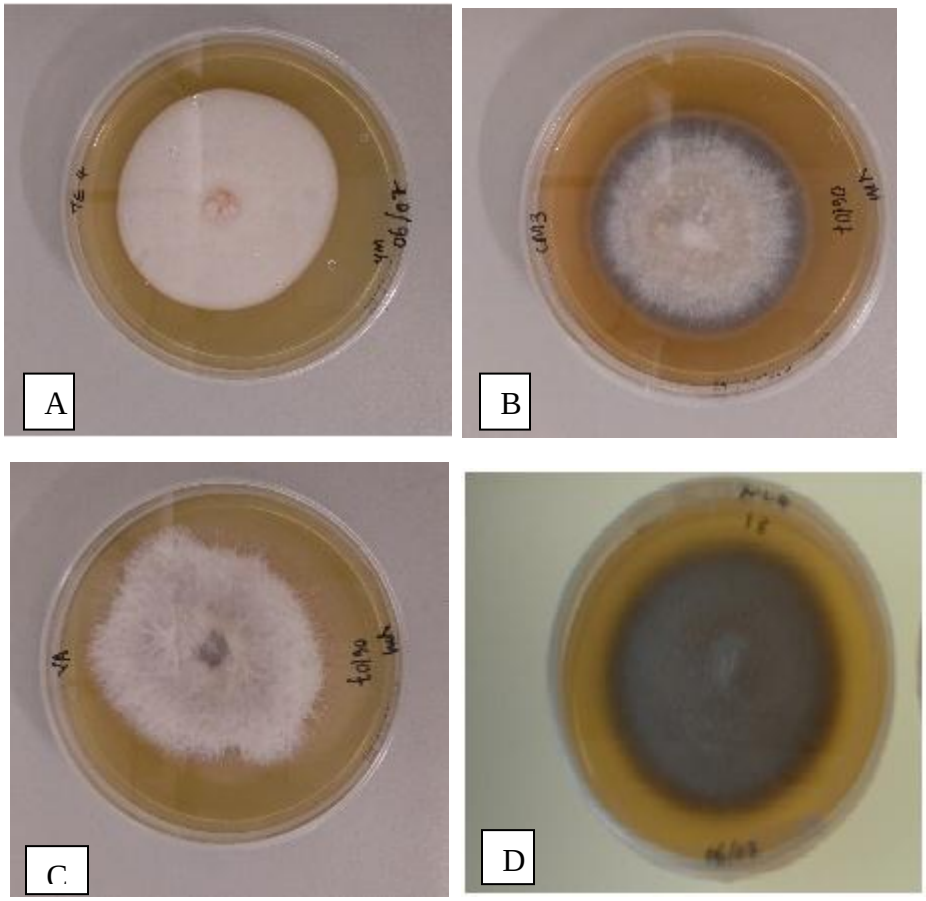


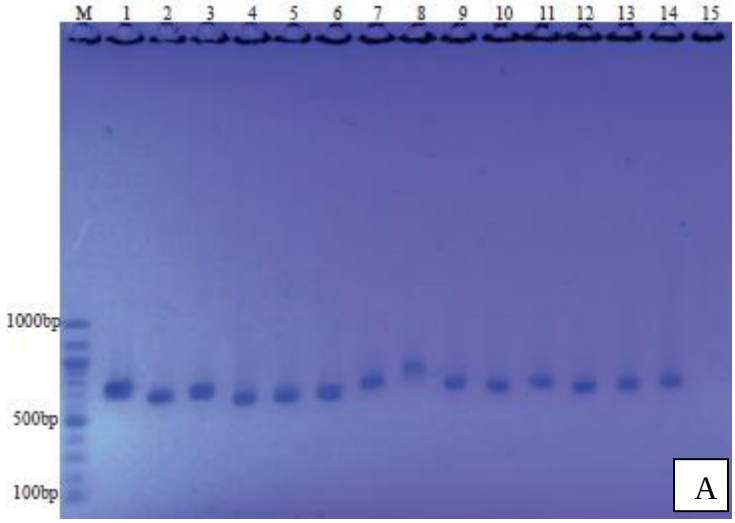
Plate 4: Pure cultures of fungal endophytes isolated from leaves of medicinal plants A- *Colletotrichum* sp B- *Fusarium* sp C- *Fusarium* sp D- *Fusarium* sp

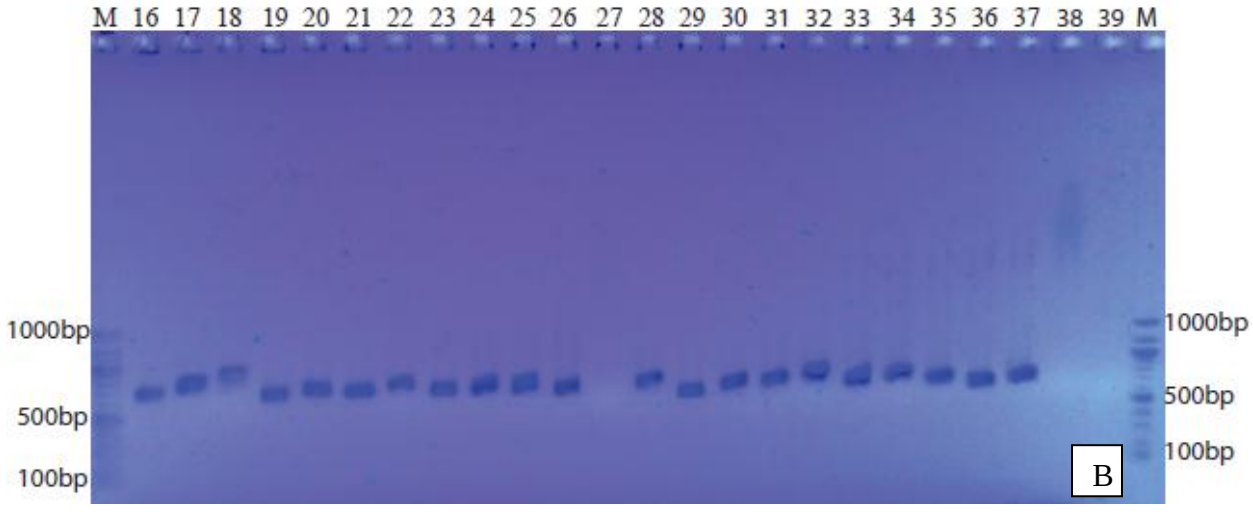
Table 1: Fungal endophytes isolation frequency and their plant sources

Plant Name and common name	**Number of isolates**
Piper capense (Wild pepper)	4
Kigelia africana (Sausage tree)	2
Tragia insuavis	3
Teclea nobilis	3
Polyscias fulva (Parasol tree)	2
Afromomum angustifolium	1
Trichilia emetica (Natal mahogany)	4
Markhamia lutea (Nile Tulip)	3
Toddalia asiatica (Orange climber)	2
Clausena anisata	5
Albizia gummifera (Smooth barked flat-crown)	3
Revolva caffra	2
Vernonia amygadalina (Bitter leaf)	1
Tithonia diversifolia	2
Barsamia alba	1
Mondia wheitei (White's ginger)	3
Warburgia ugandensis (Uganda greenheart)	4
Prunus africana (Iron wood)	3
Croton macrostachyus (Broad leaved croton)	2
Zanthoxylum gilletii (African Satinwood)	2
Brugmansia sp.	2
Erythrococca sp.	1
Total	**55**

3.4.2 DNA Extraction and PCR

The success rate of fungal endophyte DNA extraction was high because out of the 65 isolated fungal endophytes, 59 successful PCR reactions and ITS sequences were obtained. The ITS PCR products after size separation by agarose gel electrophoresis (Plate 5) clearly show that the PCR products gave the expected DNA band size (500-700bp) indicating that the primers used successfully amplified the ITS region. Lanes 1-65 represent DNA bands of the fungal endophyte isolates; however, lanes 15, 27, 38, 39, 64, and 65 had no DNA bands.





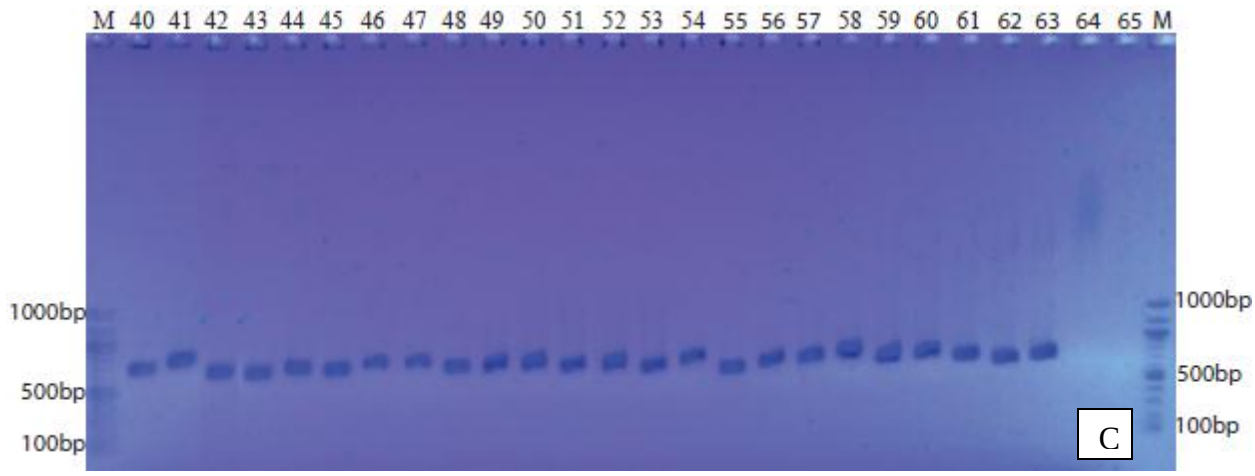


Plate 5: A, B, and C: Amplified ITS fragments of fungal endophytes isolates (lanes 1-65) observed on 0.8% Agarose gel. M- DNA Molecular ladder (100-1000bp).

3.4.3 DNA Sequencing and Phylogenetic Analyses

Data from molecular and phylogenetic analyses were used to characterize taxonomically the endophytic fungi isolated from medicinal plants. The ITS1-5.8S-ITS2 consensus sequences of the isolates were compared to ITS sequences of other organisms represented in the NCBI database GenBank. BLAST results revealed that isolated fungal endophytes belonged to three classes; *Eurotiomycetes*, *Sordariomycetes* and *Dothideomycetes* including nine genera; *Colletotrichum* (45%), *Fusarium* (32%), *Phomopsis* (7%), *Pestalotiopsis* (6%), *Aspergillus* (2%), *Chaetomium* (2%), *Cladosporium* (2%), *Trichothecium* (2%) and *Phoma* (2%) (Figure1). Results generated from ITS sequencing and phylogenetic analyses identified 47 out of 55 isolates of both sporulating and non-sporulating endophytic fungi to the genus level (Table 2). Molecular tools could not identify eight fungal endophyte isolates.

Phylogenetic analyses of the consensus sequences generated an original phylogenetic tree (Figure 2) as well as a bootstrap consensus tree (Figure 3). The generated dendogram (Figure 2) showed that the endophytic fungal isolates belonged to a diverse group of fungi distributed within the phylum Ascomycota and were dominated by three classes: *Sordariomycetes*, *Eurotiomycetes* and *Dothideomycetes*. The dendogram constituted of four major clades (clade 1: *Colletotrichum*; clade 2: *Fusarium*; Clade 3: *Phomopsis*, *Phoma*, *Pestalotiopsis*, *Cladosporium*, *Aspergillus* and *Trichothecium*; Clade 4: *Fusarium*) and one out-group (*Chaetomium*).

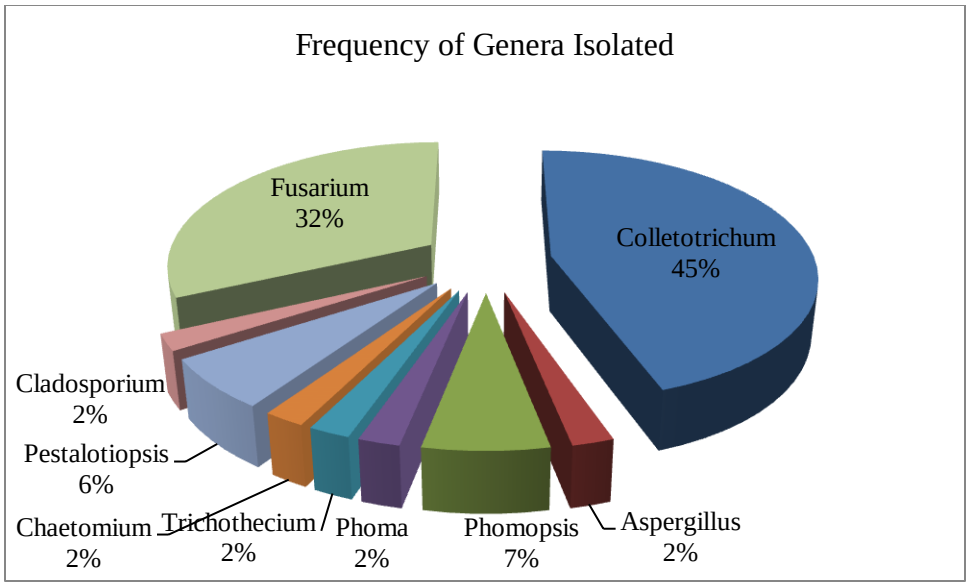


Figure 1: Relative frequencies of different fungal endophytic taxa isolated from 23 Kenyan medicinal plants

A member of the genus *Aspergillus* was identified from the *Eurotiomycetes*, while six genera were clustered under the *Sordariomycetes* and two fungal endophytes (*Cladosporium* sp. and *Phoma* sp.) were clustered in the class *Dothideomycetes*. For the three classes, BLAST analysis demonstrated high percentage similarities (Appendix 4) for different species amongst the same genera.

Table 2: Percentage sequence similarity of representative fungal endophytes from NCBI database

Fungal Isolate	**Closely related species**	**Query coverage**	**Sequence similarity %**	**Accession number**
V2	*Fusarium* sp.	100%	99%	KU984712
V24	*Chaetomium* sp.	98%	100%	KF747364
V25	*Pestalotiopsis* sp.	98%	100%	KP747698
V45	*Cladosporium* sp.	98%	100%	KX078479
V31	*Colletotrichum* sp.	97%	99%	KP942898
V20	*Aspergillus* sp.	98%	99%	KP329760
V48	*Phoma* sp.	99%	99%	FJ228201
V38	*Fusarium* sp.	98%	100%	KT313636
V33	*Colletotrichum* sp.	96%	98%	JQ754029

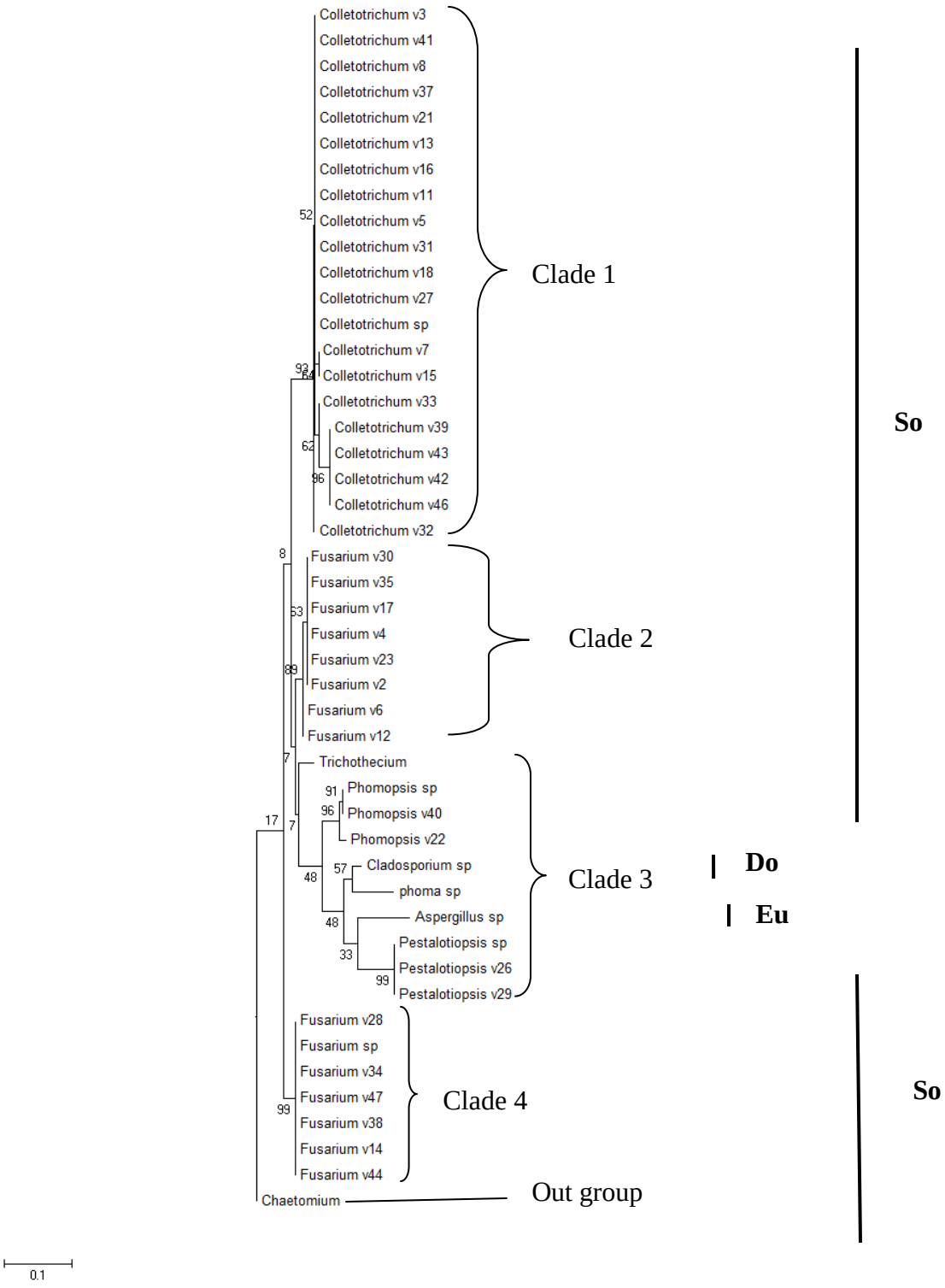


Figure 2: Phylogenetic tree of the fungal endophytes based on ITS analyses (Maximum Likelihood method; 1000 replicate bootstrap)

Class: So (Sordariomycetes), Eu (Eurotiomycetes), Do (Dothideomycetes)

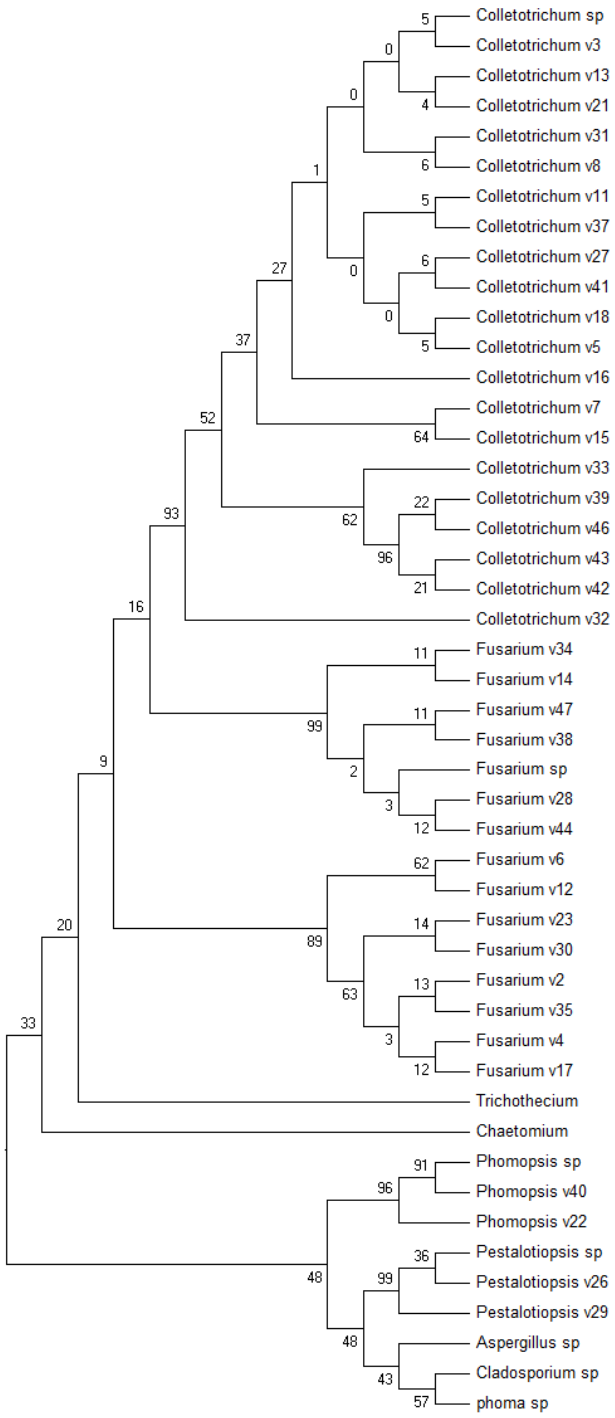


Figure 3: Bootstrap consensus tree created for testing reliability of the dataset

3.5 Discussion

The isolated fungal endophytes in this study belonged to the common isolated taxa, including *Colletotrichum*, *Pestalotiopsis*, *Phomopsis*, *Cladosporium*, *Chaetomium*, *Aspergillus*, and *Fusarium*. In the same manner, similar taxa of fungal endophytes have been previously reported as endophytes occurring in medicinal plants (Taylor *et al*., 1999; Zou *et al*., 2000; Maezza *et al*., 2003; Rakotoniriana *et al*., 2008). *Colletotrichum* and *Fusarium* were the genera that were commonly shared among the twenty-two medicinal plants. *P. capense*, *T. emetica*, and *W. ugandensis* had the highest number of fungal endophyte isolates. However, some endophytes in the genera *Phomopsis*, *Phoma*, and *Cladosporium* were isolated specifically from *T. emetica*, *M. wheitei*, and *R. caffra* respectively. This observation could mean that there might be fungal endophytes that are specifically associated with certain medicinal plants (Huang *et al*., 2008). Currently, molecular identification techniques stand out as the most effective method for identification of non-sporulating fungal endophytes and the detection of viable but non-culturable fungi (Grunig *et al*., 2002). Therefore, fungal endophytes in this study were subjected to molecular identification based on sequencing the ITS1-5.8S-ITS2 region. The latter generates a considerable sequence variation between closely related species due to a faster rate of evolution thus, sequences of these regions provide a good resolution at lower taxonomic levels (genus and species level) (Tonnabel *et al*., 2014).

Uses of ITS sequences, however, have shortcomings in that they might not achieve a perfect sequence alignment at higher taxonomic levels such as family, order and class (Lindhal *et al*., 2013) due to high ITS variability. In addition, about 20-30% of ITS sequences obtained from GenBank and other public databases for comparative analysis may not be accurate in their identification (Huang *et al*., 2009), a challenge that was equally encountered in this study as well. The drawback is attributed to the fact that most of the sequences deposited in these public databases fail to match the organism in question. Moreover, previous studies have indicated that the little ITS variation in ascomycete fungi makes the region undesirable for taxonomic identification at the species level (Skouboe *et al* 1999; Varga *et al*., 2000; Pařenicová *et al*., 2001; Lindner & Banik, 2011; Wang *et al*., 2011; Jang, 2014). Similar to this study, isolated fungal endophytes were classified under the phylum Ascomycota whose identification was achieved to the genus level.

In this study, DNA extraction and PCR amplification were successful; about 4% of the fungal endophyte, isolates were not amplified. Lack of PCR amplification could be due to some of the very likely reasons such as the bias of primers, which may amplify ITS regions towards certain groups of fungi (Bellemain *et al*., 2010). Based on our results, ITS1F and ITS4 were amplified more efficiently in the three classes from the phylum Ascomycota. Some ITS primers like ITS1 and ITS5 are usually biased towards the amplification of *Basidiomycetes* while others, such as ITS2, ITS3 and ITS4 lean towards *Ascomycetes* (Bellemain *et al*., 2010). However, in this study, other primers targeting D1/D2 and 18S rDNA regions were not used. Another reason could be probably due to production of PCR inhibitory secondary metabolites in culture (Paterson, 2004). Forty-nine PCR products were subjected to DNA sequencing, which yielded 47 readable sequences (Appendix 3) that were compared with other sequences in the NCBI database for identification. Two PCR products had no readable sequences; this could be due to errors that might have occurred during the sequencing process considering that the process is sensitive. Another reason might be weak PCR products obtained during amplification.

A BLAST analysis revealed high percentage similarity of different species among the same genera (Appendix 4), suggesting that ITS region of some isolates are conserved. A phylogenetic tree was inferred based on the consensus sequences obtained. Fungal endophytes in the genera *Pestalotiopsis* (99%), *Phomopsis* (96%), and *Fusarium* (99%) exhibited high bootstrap values, whereas *Aspergillus* (33%) and *Chaetomium* (17%) showed low bootstrap values. Bootstrap values represent phylogenetic accuracy (Felsenstein, 1985), bootstrapping test whether the dataset supports the generated tree. Values above 50% are likely to indicate reliable groupings (Hillis & Bull, 1993) while lower values mean that the node in question was found in less than half of the bootstrap replicates. In relation to the findings obtained in the current study, it can be deduced that fungal endophytes belonging to *Fusarium*, *Colletotrichum*, *Pestalotiopsis*, *Phomopsis*, *Cladosporium* and *Phoma* were reliably grouped (bootstrap values > 50%) as opposed to *Chaetomium* and *Aspergillus* (bootstrap values < 50%).

About 45% of the isolates showed sequence homology to the genera *Colletotrichum* and 32% *Fusarium* while a few sequences exhibited sequence homology with the genera *Pestalotiopsis* (6%), *Phomopsis* (7%), *Phoma* (2%), *Cladosporium* (2%), *Aspergillus* (2%), and *Chaetomium* (2%). Although a definitive taxonomic identification (at species level) of fungal

endophytes isolated in this study was not achieved, the data generated indicate great diversity of these organisms in the medicinal plants. These results therefore, show that molecular markers are an essential part of the phylogenetic analysis, and it may lead to further research on endophytes, their characterization, and assessment of their genetic diversity. Further research should focus on definitive taxonomic identification methods using molecular markers such as β-tubulin gene and elongation factor 1α gene (EF-1α) in addition to the ITS rDNA gene in molecular identification of the isolates to the species level.

References

Baldwin, B. G., Sanderson, M. J., Porter, J. M., Wojciechowski, M. F., Campbell, C. S., & Donoghue, M. J. (1995). The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden*, **82**: 247-277.

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., & Kauserud, H. (2010). ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC Microbiology*, **10**: 1-9.

Espinel, M.A., Laszlo, A., Simonsen, L., Boulahbal, F., Kim, S.J., Reniero, A., Hoffner, S., Rieder, H.L., Binkin, N., Dye, C., Williams, R. &Raviglione, M.C. (2001). Global trends in resistance to antituberculosis drugs. *The New England Journal of Medicine,* **344**: 1294–1303.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* **39**:783-791.

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology*, **2**: 113-118.

Grunig, C. R., Sieber, T. N., Rogers, S. O., & Holdenrieder, O. (2002). Genetic variability among strains of *Phialocephala fortinii* and phylogenetic analysis of the genus Phialocephala based on rDNA ITS sequence comparisons. *Canadian Journal of Botany*, **80**: 1239-1249.

Hillis, D.M. & Bull, J.J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analyses. *Systematic Biology* **42**: 182–192.

Huang, W. Y., Cai, Y. Z., Hyde, K. D., Corke, H., & Sun, M. (2007). Endophytic fungi from *Nerium oleander* L. (Apocynaceae): main constituents and antioxidant activity. *World Journal of Microbiology and Biotechnology*, **23**: 1253-1263.

Huang, W. Y., Cai, Y. Z., Hyde, K. D., Corke, H., & Sun, M. (2008). Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal diversity*, **33**: 61-75

Huang, W. Y., Cai, Y., Surveswaran, S., Hyde, K. D., Corke, H., & Sun, M. (2009). Molecular phylogenetic identification of endophytic fungi isolated from three *Artemisia* species. *Fungal Diversity*.**36**: 69-88.

Jang, J. H., Bae, S. E., Jung, S. H., Kim, H. Y., Ahn, I. S., & Son, H. S. (2014). FGMD: Database of Fungal Genetic Markers and Multigene Analysis in Fungal Classification. *The Korean Journal of Public Health* **51**: 81-89.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Mentjies, P., & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data.*Bioinformatics*, **28**: 1647-1649.

Kim, S. C., Crawford, D. J., & Jansen, R. K. (1996). Phylogenetic relationships among the genera of the *Subtribe Sonchinae* (Asteraceae): evidence from ITS sequences. *Systematic Botany*, **21**: 417-432.

Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**:111-120.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R. & Thompson, J. D. (2007). Clustal W and Clustal X version 2.0. *bioinformatics*, **23**: 2947-2948.

Lindahl, B. D., Nilsson, R. H., Tedersoo, L., Abarenkov, K., Carlsen, T., Kjøller, R., & Kauserud, H. (2013). Fungal community analysis by high-throughput sequencing of amplified markers–a user's guide. *New Phytologist*, **199**: 288-299.

Lindner, D. L., & Banik, M. T. (2011). Intragenomic variation in the ITS rDNA region obscures phylogenetic relationships and inflates estimates of operational taxonomic units in genus *Laetiporus*. *Mycologia*, **103**: 731-740.

Meazza, G., Dayan, F. E., & Wedge, D. E. (2003). Activity of quinones on *Colletotrichum* species. *Journal of Agricultural and Food chemistry*, **51**: 3824-3828.

Pařenicová, L., Skouboe, P., Frisvad, J., Samson, R. A., Rossen, L., ten Hoor-Suykerbuyk, M., & Visser, J. (2001). Combined molecular and biochemical approach identifies *Aspergillus japonicus* and *Aspergillus aculeatus* as two species. *Applied and Environmental Microbiology*, **67**: 521-527.

Paterson, R.R.M. (2004). The isoepoxydon dehydrogenase gene of patulin biosynthesis in cultures and secondary metabolites as candidate PCR inhibitors. *Mycological Research*, **108**: 1431-1437.

Peay, K. G., Kennedy, P. G., & Bruns, T. D. (2008). Fungal community ecology: a hybrid beast with a molecular master. *Bioscience*, **58**: 799-810.

Petrini, O., T. N. Sieber, L. Toti and O. Viret. (1992). Ecology, metabolite production, and substrate utilization in endophytic fungi. *Natural Toxins*, **1**: 185-196.

Pillay, D. and Zambon, M. (1998). Antiviral drug resistance. *British Medical Journal*, **317**: 660–662.

Rakotoniriana, E. F., Munaut, F., Decock, C., Randriamampionona, D., Andriambololoniaina, M., Rakotomalala, T., & Mahillon, J. (2008). Endophytic fungi from leaves *of Centella asiatica*: occurrence and potential interactions within leaves. *Antonie van Leeuwenhoek*, **93**: 27-36.

Skouboe, P., Frisvad, J. C., Taylor, J. W., Lauritsen, D., Boysen, M., & Rossen, L. (1999). Phylogenetic analysis of nucleotide sequences from the ITS region of terverticillate *Penicillium* species. *Mycological Research*, **103**: 873-881.

Tamura K., Stecher G., Peterson D., Filipski A., & Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, **30**: 2725-2729.

Taylor, J. W., Jacobson, D. J., & Fisher, M. C. (1999). The evolution of asexual fungi: reproduction, speciation and classification. *Annual Review of Phytopathology*, **37**: 197-246.

Tonnabel, J., Olivieri, I., Mignot, A., Rebelo, A., Justy, F., Santoni, S., Caroli, S., Sauné, L., Bouchez, O. & Douzery, E. J. (2014). Developing nuclear DNA phylogenetic markers in

the angiosperm genus Leucadendron (Proteaceae): A next-generation sequencing transcriptomic approach. *Molecular Phylogenetics and Evolution*, **70**: 37-46.

Varga, J., Tóth, B., Rigó, K., Téren, J., Hoekstra, R. F., & Kozakiewicz, Z. (2000). Phylogenetic analysis of *Aspergillus* section Circumdati based on sequences of the internal transcribed spacer regions and the 5.8 S rRNA gene. *Fungal Genetics and Biology*, **30**: 71-80.

Wang, H., Xiao, M., Kong, F., Chen, S., Dou, H. T., Sorrell, T., & Xu, Y. C. (2011). Accurate and practical identification of 20 *Fusarium* species by seven-locus sequence analysis and reverse line blot hybridization, and an *in vitro* antifungal susceptibility study. *Journal of Clinical Microbiology*, **49**: 1890-1898.

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: A Guide to Methods and Applications*, **18**: 315-322.

Zinniel, D. K., Lambrecht, P., Harris, N. B., Feng, Z., Kuczmarski, D., Higley, P., & Vidaver, A. K. (2002). Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Applied and Environmental Microbiology*, **68**: 2198-2208.

Zou, W. X., Meng, J. C., Lu, H., Chen, G. X., Shi, G. X., Zhang, T. Y., & Tan, R. X. (2000). Metabolites of *Colletotrichum gloeosporioides*, an endophytic fungus in *Artemisia mongolica*. *Journal of Natural Products*, **63**: 1529-1530.

CHAPTER FOUR

BIOACTIVITY OF ENDOPHYTIC FUNGI AGAINST SELECTED HUMAN DISEASE CAUSING PATHOGENS

4.1 Abstract

Antibiotic resistance remains a global health threat. This challenge has prompted scientists to go back to natural product studies with the hope of finding new lead compounds that can be used in the development of effective and affordable pharmaceuticals. This study aimed to screen for antimicrobial activities of fungal endophytes isolated from fresh leaves of selected medicinal plants. Antagonism studies were done by carrying out a dual culture assay where plugs of endophytic fungal isolates were plated in Mueller Hinton agar seeded with test bacteria. Eighteen fungal endophyte isolates (30.0%) demonstrated antibacterial activity (inhibition zone >14 mm) against gram-positive bacterium *S. aureus* with isolates in the genera *Colletotrichum* and *Fusarium* being the most active. *Colletotrichum* sp. exhibited the largest inhibition zone of 17.7±1.20 mm. Twenty five fungal endophyte isolates (41.7%) showed antibacterial activity (>14 mm) against gram negative bacterium *P. aeruginosa* with *Colletotrichum* sp. registering maximum activity of inhibition diameter 17.00±1.15 mm. Metabolic extracts from *Phomopsis* sp. and *Colletotrichum* sp. recorded maximum inhibition diameters of 27 mm against *B. subtilis* compared to chloramphenicol (30 mm). Metabolic extracts from *Phomopsis* sp. also demonstrated activity against *K. pneumoniae* (12 mm). No activity was observed against *P. aeruginosa* and *K. pneumonia* with extracts from *Chaetomium* sp. and *Colletotrichum* sp. Extracts from *Aspergillus* sp. were active at low concentrations against *B. subtilis* (2.34 µg/ml) and *C. tenius* (9.38 µg/ml). These results suggest that medicinal plants harbor fungal endophytes capable of producing bioactive secondary metabolites that can be exploited for their antimicrobial potential.

4.2 Introduction

Endophytes are a group of microorganisms, either bacteria or fungi, that reside in tissues of healthy plants, inter and intracellularly without causing any disease symptom (Wilson, 1995). They form a rich and powerful untapped source of novel natural products for exploitation in agriculture, medicine and industry since their discovery (Yu *et al*., 2010). Endophytes are present

in all plant species and contribute to their plant host by providing protection to the plant against pathogenic infections by producing antimicrobial compounds as secondary metabolites and in return, the host plant provides these endophytes with nutrients (symbiosis) (Yu *et al*., 2010). The relationship between endophytes and host plants may range from latent phytopathogenesis to mutualistic symbiosis (Sturz *et al*., 1997). Studies have demonstrated that medicinal plants form a rich reservoir of novel bioactive secondary metabolites (Weber *et al*., 2004; Tejesvi *et al*., 2006; Aly *et al*., 2008; Sappapan *et al*., 2008). Extensive research on secondary metabolites production in medicinal plants has shown that endophytic fungi inhabiting medicinal plants play a significant role in the production of these metabolites, which have antibacterial, antifungal, anti-cancer, and antioxidant activities (Arnold *et al*., 2003). Hence, medicinal plants are a rich source of fungal endophytes, which produce compounds of pharmaceutical value (Strobel *et al*., 2004; Tejesvi *et al*., 2007).

Studies have been done on fungal endophytes from various plant sources in an attempt to isolate and identify novel bioactive compounds of pharmaceutical importance. As a result, many novel compounds with outstanding antimicrobial, cytotoxic, antitumor, anti-inflammatory, antiviral and anticancer activities have been discovered. These compounds are mainly categorized as quinones, isocoumarins, lactones, terpenoids, phenylpropanoids, phenols and alkaloids (Zhang *et al*., 2006; Zhou *et al*., 2009 and Yu *et al*., 2010). Paclitaxel was found to be active against some tumors including, lung, head, breast and ovarian tumors. Podophyllotoxin, a precursor of several anti-cancer drugs, was recently discovered to be produced by the fungal endophyte *Fusarium oxysporum*, which occurs naturally as an endophyte in the medicinal plant *Juniperus recurva* (Kour *et al*., 2008). The main aim of this study was to screen for antimicrobial activities of fungal endophytes isolated from leaves of selected Kenyan medicinal plants

4.3 Materials and Methods

4.3.1 Media Preparation for Culturing, Fermentation and antimicrobial assays

For fungal microorganism, Yeast malt 6.3 media (glucose 4g/l, yeast extract 4g/l, malt extract 10 g/l and agar 20 g/l and pH 6.3) (Stadler *et al.*, 2004) and PDA (Potato infusion 200g/l, Dextrose 20g/l, Agar 15g/l and pH 5.6) were used. For bacteria EBS (casein peptone M 5 g/l, D-glucose 5 g/l, meat extract 1 g/l, kat yeast extract 1 g/l, 50mM HEPES buffer 11 g/l, ph 7.0) was used. Fermentation media included yeast malt medium (glucose 4g/l, yeast extract 4g/l, malt

extract 10g/l pH 6.3) and sugar-malt medium (molasses 5g/l, oatmeal 5g/l, saccharose 4g/l, mannitol 5g/l, glucose 1.5g/l, $CaCO_3$ 1.5g/l, Edamin 0.5g/l, $(NH_4)2SO_4$ 0.5g/l pH 7.2), which was used to cultivate endophytic fungi isolates in a shake flask system. Media was autoclaved at 121ºC and 15pa for 15 minutes.

4.3.2 Antagonistic Screening of Fungal Endophytes against Test Microorganisms

Antagonistic screening of endophytic fungal isolates was done using the dual culture assay following the method described by Stadler *et al*. (2004). The endophytic isolates were grown on PDA medium for 20 days at 25± 2ºC. Plugs of approximately 7 mm were cut using a sterile cork borer and placed in Mueller Hinton agar plates that were seeded with 10^5 CFU/ml *S. aureus* ATCC25922 (gram positive bacteria) and *P. aeruginosa* ATCC87853 (gram negative bacteria). To allow for diffusion of secondary metabolites into the agar, the agar plates were stored at 4ºC for 24 h and subsequently incubated at 37ºC overnight. Inhibition zones were measured after 24 h and isolates that demonstrated antimicrobial activity (>10 mm) were selected for further secondary screening. The experiment was done in triplicate.

4.3.3 Fermentation and Extraction of Fungal Endophyte Extracts

The inocula were prepared by introducing 7 mm mycelial agar plugs from 10-days-old fungal culture into 250 mL Erlenmeyer flasks containing 200 mL sugar malt and yeast malt broth medium. The cultures were cultivated at 23ºC with a rotational speed of 140 rpm. After 10-14 days of incubation, the fungal biomass and the fermentation broth were separated by filtration. The fungal biomass was extracted by soaking in 200 mL acetone followed by mixing on a magnetic stirrer for 5 minutes and then subjected to an ultrasonic sound bath for 30 min. The fungal biomass was separated from acetone by filtration, then the filtrate which constituted acetone was concentrated by evaporation on a vacuum rotavapor at 40ºC and pressure 540pa leaving behind the water phase. An equal volume of ethyl acetate was added to the water phase, and the upper organic phase was then concentrated to dryness. The dry extract obtained was weighed and stored at -20ºC for further analysis. The final pH of the fermentation broth was measured followed by extraction with equal volume of ethyl acetate. The upper organic phase was concentrated to dryness on a vacuum rotavapor at 40ºC and a pressure of 240pa. The dry extract obtained was weighed and stored at -20ºC for further analysis.

4.3.4 Preliminary Screening of Crude Fungal Endophyte Extracts

Submerged cultures of selected eight bioactive endophytic fungi were prepared by introducing 7 mm mycelial plugs in 500ml Erlenmeyer flasks each containing sterilized 250ml M2 medium (5g/l malt extract, 2g/l yeast extract and 2g/l glucose pH 7.4) (Matasyoh *et al*., 2011). The cultures were incubated for 21 days at room temperature (17±2ºC) in a dark room. The culture broths were filtered through Whatman filter paper yielding the supernatant and fungal biomass separately. Secondary metabolites from the supernatant were extracted three times with ethyl acetate by solvent extraction procedure. An equal volume of the filtrate (aqueous phase) and ethyl acetate was placed in a separating funnel and hand-shaken gently for 10 min to avoid emulsion formation. The organic phase was passed through anhydrous sodium sulphate to remove water traces and after that, it was evaporated to dryness in a vacuum rotary evaporator (Buchi Rotavapor) at a rotation of 50 rpm and a vapor temperature of 40ºC. Crude extracts obtained were weighed and stored at 4ºC. Sterile blank Whatman discs, impregnated with 100 µL of ethyl acetate extract with a concentration of 1 mg/mL, were then placed on the surface of MH media seeded with 10^5 CFU/ml of *B. subtilis*, *S. aureus, E. coli, K. pneumoniae,* and *P. aeruginosa*. The negative control used in this experiment was 1% dimethyl sulfoxide (DMSO) whereas 30 µg/disc chloramphenicol discs were used as the positive control. The plates were incubated at 37ºC for 24 hours and thereafter diameters of inhibition zones were measured.

4.3.5 Minimum Inhibitory Concentration (MIC) test

Antimicrobial activity of fungal endophyte extracts was determined using the serial dilution assay test as described by Okanya *et al.* (2011). Overnight 24 h cultures of *B. subtilis* DSM10, and *E. coli* DSM498 were prepared by inoculating 1ml of the stock cultures in sterile 100 ml EBS media. The cultures were then incubated at 30ºC for 24 h. Fungal cultures of *C. tenius* MUCL29892 and *M. plumbeus* MUCL4935 were prepared by inoculating 1ml of the stock cultures in sterile 100 ml of yeast malt media and the cultures were then incubated at 23ºC for 48 hours. Bacterial cell suspensions were diluted to 10^5 CFU/ml and 280 µl of each cell suspension was pipetted into the first row (A1-A12) of a 96-well plate. In addition, 20 µl of 4.5 mg/ml crude fungal endophyte extracts was pipetted into the first row to make a final concentration of 300µg/ml. Thereafter, a 2-fold serial dilution with concentrations ranging 300-2.34 µg/ml was made. Cycloheximide and nystatin were used as reference antibiotic and antifungal, respectively with concentrations ranging 100-0.78µg/ml. The 96-well plates with bacteria and extracts were

incubated at 30ºC for 24 h, whereas plates with fungal pathogens with extracts were incubated at 23ºC for 48 h. The presence of clear wells was used as an indicator of antimicrobial activity. Strains which showed antimicrobial activity at low concentrations were selected for further analysis. Minimum Inhibitory Concentration was determined as the lowest concentration of fungal endophyte extract that inhibited visible growth.

4.3.6 Data Analysis

Mean inhibition zones were calculated and equality of means was analyzed using one-way analysis of variance (ANOVA). Tukey's Honestly Significant Difference (HSD), a Post-Hoc Analysis, was used to determine if there was any significant difference between the means of the isolates. Data analysis was performed using R statistical software version 3.3.1.

4.4 Results

4.4.1 Antagonistic Assay of Fungal Endophytes against Test Human Pathogenic Bacteria

The fungal endophytes were tested for antagonism by the dual culture assay method against pathogenic bacteria, *S. aureus* ATCC25922 and *P. aeruginosa* ATCC87853 (Plate 6). Eighteen endophytic fungal isolates (30.0%) demonstrated antagonism ($P< 0.01$) against *S. aureus* ATCC25923 (Table 3). This activity was not significantly different from the positive control (chloramphenicol) with inhibition zone >14 mm. Endophytic fungal isolates in the genera *Pestalotiopsis, Colletotrichum* and *Fusarium* were the most active; *Colletotrichum* sp. exhibited a maximum inhibition zone of 17.7±1.20 mm. In addition, antagonism against *P. aeruginosa* ATCC87853 of twenty five endophytic fungal isolates (41.7%) was not significantly different ($P< 0.01$) from the positive control (Appendix 1). *Colletotrichum* sp., *Pestalotiopsis* sp. and *Fusarium* sp. were the most active with inhibition zone diameter >14 mm. Maximum activity was registered by *Colletotrichum* sp. with an inhibition diameter of 17.0±1.15 mm. Isolates in the genera *Phomopsis, Chaetomium*, and *Cladosporium* showed minimal antibacterial activity with significant differences in their means with the positive control. Some members of *Fusarium* and *Colletotrichum* were inactive against the two bacteria.

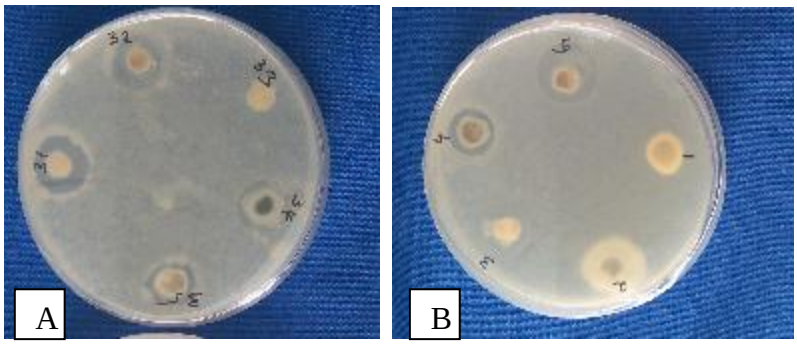


Plate 6: Antagonistic test of endophytic fungi against *Staphylococcus aureus* (A) and *Pseudomonas aeruginosa* (B)

4.4.2 Preliminary Disc Diffusion Assay of Fungal Endophyte Crude Extracts

Basing on the antagonism demonstrated by the fungal endophytes in the primary screening assay, seven fungal endophytes were subjected to submerged fermentation and extraction of secondary metabolites. The extracts were then subjected to disc diffusion assay test and inhibition zones were measured and recorded (Table 4). Ethyl acetate extracts of *Phomopsis* sp., *Fusarium* sp. and *Colletotrichum* sp. demonstrated antibacterial activity with inhibition zones of 27 mm, 26 mm and 27 mm, respectively, against *B. subtilis* and 22 mm, 21 mm and 20 mm against *S. aureus*. These extracts also showed activity against *E. coli* with inhibition diameters of 25 mm, 20 mm, and 21 mm, respectively. Gram-negative *P. aeruginosa* and *K. pneumoniae* proved to be more resistant to the extracts showing low inhibition diameters of 8-12 mm.

Table 3: Inhibition diameters (mm) for the fungal endophytes against test organisms

	Test organisms (diameter in mm, n=3)	
Endophyte/Treatment	***Staphylococcus aureus***	***Pseudomonas aeruginosa***
Pestalotiopsis sp. (TD1)	15.67 ± 0.88^{abcde}	10.33 ± 0.33^{defghijk}
Colletotrichum sp. (RC1)	10.00 ± 0.58^{bcdefgh}	17.00 ± 1.15^{ab}
Colletotrichum sp. (KA1)	14.00 ± 1.73^{abcdefgh}	$13.00\pm0.58^{bcdefghijk}$
Aspergillus sp. (AG2)	6.33 ± 3.18^{efghi}	7.67 ± 1.20^{jk}
Colletotrichum sp. (TN2)	9.67 ± 0.33^{bcdefgh}	14.00 ± 1.00^{abcdefgh}
Phomopsis sp. (KA2)	4.67 ± 2.60^{hi}	8.00 ± 0.58^{ijk}
Colletotrichum sp. (WU3)	13.00 ± 1.53^{bcdefgh}	0.00 ± 0.00^{l}
Pestalotiopsis sp. (BA2)	12.67 ± 2.40^{bcdefgh}	15.33 ± 0.33^{abcde}
Colletotrichum sp. (CA2)	11.67 ± 0.33^{bcdefgh}	13.33 ± 1.67^{bcdefghij}
Phomopsis sp. (TE2)	13.00 ± 1.00^{bcdefgh}	9.33 ± 1.86^{fghijk}
Fusarium sp. (WU2)	13.00 ± 0.58^{bcdefgh}	0.00 ± 0.00^{l}
Chloramphenicol	22.47 ± 0.07^{a}	19.67 ± 0.88^{a}
Negative Control (DMSO)	0.00 ± 0.00^{i}	0.00 ± 0.00^{l}

Within a column, fungal endophytes sharing the same letter(s) are not significantly different while those with different letter (s) are significantly different (α =0.05, Tukey's test).

Table 4: Inhibition diameters (mm) of Ethyl acetate extracts of selected fungal endophytes against test bacteria

Isolate code	Endophyte	Test organisms (diameter in mm)				
		B.S	**S.A**	**E.C**	**K.P**	**P.A**
TE2	*Phomopsis* sp.	27	22	25	12	8
AG2	*Aspergillus* sp.	8	0	7	0	11
TE1	*Chaetomium* sp.	19	13	13	0	0
PF2	*Fusarium* sp.	0	0	0	0	11
WU1	*Colletotrichum* sp.	0	0	0	0	0
WU2	*Fusarium* sp.	26	21	20	8	12
WU3	*Colletotrichum* sp.	27	20	21	11	10
ML2	*Fusarium* sp.	8	0	6	0	7
Chloramphenicol		30	27	26	15	18
1% DMSO		0	0	0	0	0

B.S- *B. subtilis*; S.A- *S. aureus*; E.C- *E. coli*; KP- *K. pneumoniae*; PA- *P. aeruginosa*

4.4.3 Minimum Inhibitory Concentration (MIC) test

This study went on further to perform serial dilution assay test to screen for antimicrobial activity of selected fungal endophyte extracts and MIC determination (Plate 7). Antimicrobial activity of mycelium and supernatant ethyl acetate extracts of the selected isolates were tested (Table 5). Ethyl acetate extracts of fungal endophyte isolates including *Colletotrichum* sp., *Pestalotiopsis* sp. and *Phomopsis* sp. showed antimicrobial activity (indicated by clear wells in Plate 7) against *B. subtilis* DSM10, *E. coli* DSM498, *C. tenius* MUCL29892 and *M. plumbeus* MUCL4935. Supernatant extracts of *Pestalotiopsis* sp. isolated from *Tithonia diversifolia* showed antibacterial activity against *B. subtilis* at high concentrations with MIC values of 75µg/ml and 150µg/ml. No activity was observed against *E. coli*, *C. tenius* and *M. plumbeus*.

Extracts of *Aspergillus* sp. isolated from *Albizia gummifera* stood out as the most effective against all the four test microorganisms. Supernatant extract was active against *B. subtilis* at low concentrations with MIC value of 9.38µg/ml in YM6.3 media and a value of 2.34µg/ml in SM1/2 media. Mycelium extract of the same isolate against *C. tenius* recorded MIC value of 9.38µg/ml. In contrast, antibacterial activity of the same extracts against *E. coli* was demonstrated at high concentrations of 75µg/ml (Table 5). The same case was observed with antifungal activity at high concentrations (150µg/ml) against *M. plumbeus*.

The genus *Colletotrichum* sp. isolated from *Kigelia africana* demonstrated antibacterial activity against *B. subtilis* at a high concentration of 300µg/ml with supernatant extracts from SM1/2 media. In contrast, supernatant extracts from YM6.3 media were active against *B. subtilis* at much lower concentration recording MIC value of 37.5µg/ml. No activity was observed against *E. coli*, *C. tenius* and *M. plumbeus*. *Phomopsis* sp. isolated from *T. emetica* was active against both *B. subtilis* and *M. plumbeus* at high concentrations of 75µg/ml and 150µg/ml (YM6.3 media) and 150µg/ml (YM6.3 media) respectively. There was no activity observed against *C. tenius* and *E. coli*.

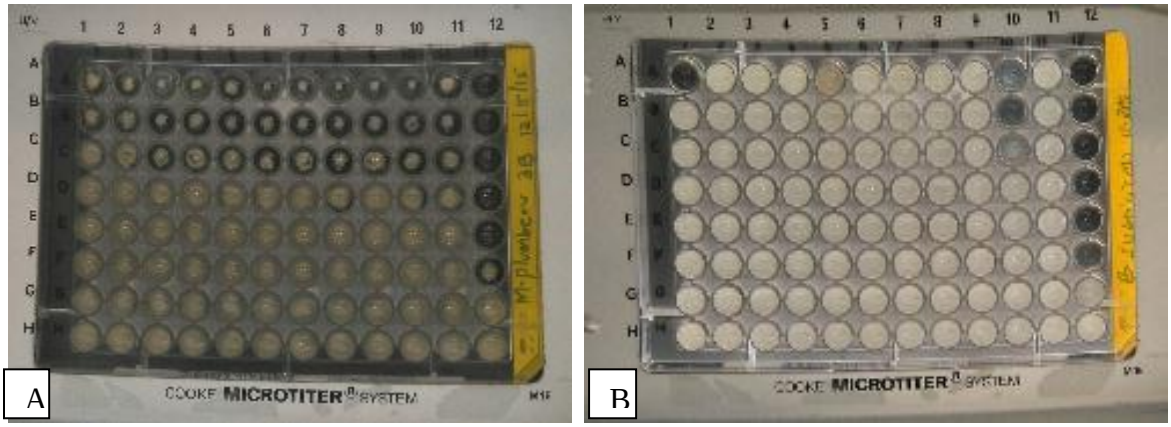


Plate 7: 96-well plate showing antimicrobial activities of fungal endophyte extracts against *M. plumbeus* (A) and *B. subtilis* (B). (Negative control, row 11; Positive control, row 12)

Table 5: Activities of fungal endophyte extract against selected test bacteria and fungi

				Extract activity (µg/mL)			
Plant source	**Endophyte**	**Media**	**E**	*B. subtilis*	*E. coli*	*M. plumbeus*	*C. tenius*
Tihonia diversifolia	*Pestalotiopsis* sp.	YM 6.3	S	150	*	*	*
			M	*	*	*	*
		SM1/2	S	75	*	*	*
			M	*	*	*	*
Kigelia africana	*Colletotrichum* sp.	YM 6.3	S	37.5	*	*	*
			M	*	*	*	*
		SM1/2	S	300	*	*	*
			M	*	*	*	*
Albizia gummifera	*Aspergillus* sp.	YM 6.3	S	9.38	75	*	150
			M	2.34	*	*	*
		SM1/2	S	2.34	18.75	150	75
			M	2.34	75	*	9.38
Trichilia emetica	*Phomopsis* sp.	YM 6.3	S	75	*	150	*
			M	150	*	*	*
		SM1/2	S	150	*	*	*
			M	150	*	*	*
Cyclohexi mide				3.13	0.78	N/A	N/A
Nystatin				N/A	N/A	6.25	3.13
Methanol (99%)				*	*	*	*

E- Extracts; S- Supernatant; M- Mycelia; (*) no activity YM-yeast malt; SM-sugar malt

4.5 Discussion

The major isolates that demonstrated maximum antimicrobial activity in culture included *Colletotrichum* sp., *Pestalotiopsis* sp. and *Fusarium* sp. Similarly, bioactive compounds

produced by endophytes growing in cultures have been found to be active against human pathogenic microorganisms (Castillo *et al*., 2002). Antagonism might be due to the production of biologically active secondary metabolites in culture media. Fungi in the genera *Fusarium* are known to be producers of mycotoxins harmful to both human and animal health (Desjardins & Proctor, 2007). Extracts from *Fusarium* spp. isolated as endophytes have been reported to demonstrate antimicrobial activity against a range of pathogenic microorganisms. Most of these biologically active extracts are composed of mycotoxins (Xu *et al*., 2010; Wang *et al*., 2011). However, in a few cases, there has been documentation of non-mycotoxic secondary metabolites that are active against pathogenic microorganisms (Tegos *et al*., 2002; Wang *et al*., 2014). As observed in this study, bioactivity of extracts from *Fusarium* sp. could possibly be due to mycotoxins or such non-mycotoxic secondary metabolites.

Fungal endophytes in the genera *Chaetomium*, *Cladosporium*, and *Phomopsis* showed minimum activity in culture. In contrast, fungal endophytes *Cladosporium* sp., from the medicinal plant *Kigelia Africana* were reported to demonstrate relatively high antimicrobial activity against selected human pathogenic microorganisms (ldris *et al*., 2013). The minimum activity of *Chaetomium* sp. observed in the current study could be due to production of low concentrations of secondary metabolites in culture. In this study, *Aspergillus* sp. was isolated as an endophyte. Similarly, several studies with many plant species, including medicinal plants have reported isolation of the same as endophytes (Bazerra *et al*., 2013, 2013; Kusari *et al*., 2013). Extracts of *Aspergillus* sp. inhibited the growth of test microorganisms at low concentrations of 2.34µg/ml against *B. subtilis*, and 9.38µg/ml against *M. plumbeus*. *Aspergillus* spp. have been reported to be prolific producers of mycotoxins that are known to be harmful to human health (Qiao *et al*., 2010). There have been reports of antimicrobial activity of extracts from *Aspergillus* sp. and compounds classified as tremorgenic mycotoxins have been identified from the endophytic fungus *Aspergillus* sp. Some of these compounds were reported to have potent antibacterial activity against *E. coli* (Qiao *et al*., 2010). Other studies have documented bioactive secondary metabolites with structures similar to Ochratoxin A moiety, one of the most abundant mycotoxins found in food (Moore *et al*., 1972). Concerning these reports, this could possibly mean that the bioactivity demonstrated by this fungal endophyte in our study, could be due to the high production of mycotoxins as secondary metabolites.

Fungal endophytes in the genus *Colletotrichum*, *Pestalotiopsis*, and *Phomopsis* demonstrated antibacterial activity against *B. subtilis*, *M. plumbeus* and *C. tenius* at high concentrations with MIC values ranging 300-37.5µg/ml. The low activity of these extracts might be attributed to low concentrations of bioactive compounds in the metabolic extracts. There have been reports of *Colletotrichum* sp. producing secondary metabolites with antimicrobial activity against pathogenic bacteria (Chapla *et al*., 2014). For example, extracts from *Colletotrichum* sp. have been documented to show antibacterial activity against *B. subtilis*, *S. aureus* (Zou *et al*., 2000) and *E. coli* (Zhang *et al*., 2009). In consonance with our studies, research done by Gomes-Figueiredo *et al.* (2007) demonstrated that the metabolic extracts produced by endophytes in the genus *Pestalotiopsis*, isolated from medicinal plant *Maytenus ilicifolia*, have antimicrobial activity against a variety of human disease causing pathogens. In addition, secondary metabolites extracted from the same fungal endophyte were reported to be active against *S. aureus* (Ding *et al*., 2009). *Phomopsis* sp. isolated in this study showed antimicrobial activity against *B. subtilis*, *S. aureus* and *E. coli* in the disc diffusion assay test. In agreement with this, secondary metabolites isolated from *Phomopsis* sp. showed antibacterial activity against *B. subtilis* and *S. aureus* (Isaka *et al*., 2001; Wagenaar and Clardy, 2001; Lim *et al*., 2010; Erbert *et al*., 2012).

In the present study, disc diffusion assay test showed that extracts from *Chaetomium* sp. demonstrated antibacterial activity against *B. subtilis*, *S. aureus*, and *E. coli*; with inhibition zones of 19, 13 and 13 mm, respectively. Fungal endophytes in the genus *Chaetomium* are widely known for the production of bioactive secondary metabolites (Kanokmedhakul *et al*., 2002). Similarly, Momesso *et al.* (2008) reported to have isolated bioactive secondary metabolites from the same fungal endophyte, which demonstrated antibacterial activity against *S. aureus*. Moreover, secondary metabolites isolated from the fungal endophyte *Chaetomium* sp. demonstrated antibacterial activity against *S. aureus* as reported by (Casella *et al*., 2013).

Members in the classes *Sordariomycetes* (*Colletotrichum* sp. and *Fusarium* sp.) and *Eurotiomycetes* (*Aspergillus* sp.) showed high functional versatility with some isolates demonstrating antimicrobial activity against test microorganisms. Differences in functional characteristics were evident among isolates of the same species for their ability to produce secondary metabolites, which are in accordance with studies done by Pelaez *et al.* (1998). Observed differences in isolates of the same species to produce secondary metabolites can be

attributed to high variability among the strains as branch nodes on the dendogram were supported by low bootstrap values indicating horizontal gene transfer (Hampl *et al*., 2001) and genetic variability as possible mechanisms for adapting to the host system.

The results obtained from this work reveal that extracts from *Colletotrichum* sp., *Phomopsis* sp., *Chaetomium* sp., *Pestalotiopsis* sp., and *Cladosporium* sp. have the potential to be exploited as leads for drug development. Previous studies indicate that natural products from fungal endophytes have been observed to have antifungal, antibacterial, antiviral, anticancer, and antiprotozoal activity (Strobel *et al*., 1999). It is evident that extracts from the mycelia demonstrated lower antimicrobial activity when compared to extracts from the supernatant fraction. This could be attributed to higher accumulation of secondary metabolites in the liquid fraction than in the solid mycelia during fermentation.

References

Aly, A. H., Edrada-Ebel, R., Indriani, I. D., Wray, V., Müller, W. E., Totzke, F., & Proksch, P. (2008). Cytotoxic metabolites from the fungal endophyte *Alternaria* sp. and their subsequent detection in its host plant *Polygonum senegalense*. *Journal of Natural Products*, **71**: 972-980.

Arnold, A. E., Mejía, L. C., Kyllo, D., Rojas, E. I., Maynard, Z., Robbins, N., & Herre, E. A. (2003). Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences*, **100**: 15649-15654.

Bazerra, J. D. P., Santos, M. G. S., Svedese, V. M., Lima, D. M. M., Fernandes, M. J. S., Paiva, L. M., & Souza-Motta, C. M. (2012). Richness of endophytic fungi isolated from *Opuntia ficus-indica* Mill. (Cactaceae) and preliminary screening for enzyme production. *World Journal of Microbiology and Biotechnology*, **28**: 1989-1995.

Casella, T. M., Eparvier, V., Mandavid, H., Bendelac, A., Odonne, G., Dayan, L., & Stien, D. (2013). Antimicrobial and cytotoxic secondary metabolites from tropical leaf endophytes: isolation of antibacterial agent pyrrocidine C from *Lewia infectoria* SNB-GTC2402. *Phytochemistry*, **96**: 370-377.

Castillo, U.F., Strobel, G.A., Ford, E.J., Hess, W.M., Porter, H., Jensen, J.B., Albert, H., Robison, R., Condron, M.A.M., Teplow, D.B., Stevens, D., Yaver, D. (2002).

Munumbicins, wide-spectrum antibiotics produced by *Streptomyces* NRRL 30562 endophytic on *Kennedia nigriscans*. *Microbiology*, **148**: 2675– 2685.

Chapla, V. M., Zeraik, M. L., Leptokarydis, I. H., Silva, G. H., Bolzani, V. S., Young, M. C. M., & Araújo, A. R. (2014). Antifungal Compounds Produced by *Colletotrichum gloeosporioides*, an Endophytic Fungus from *Michelia champaca*. *Molecules*, **19**: 19243-19252.

Desjardins, A. E., & Proctor, R. H. (2007). Molecular biology of *Fusarium* mycotoxins. *International Journal of Food Microbiology*, **119**: 47-50.

Ding, G., Li, Y., Fu, S., Liu, S., Wei, J., & Che, Y. (2009). Ambuic Acid and Torreyanic Acid Derivatives from the Endolichenic Fungus *Pestalotiopsis* sp. *Journal of Natural Products*, **72**: 1368-1368.

Erbert, C., Lopes, A. A., Yokoya, N. S., Furtado, N. A., Conti, R., Pupo, M. T., & Debonsi, H. M. (2012). Antibacterial compound from the endophytic fungus *Phomopsis longicolla* isolated from the tropical red seaweed *Bostrychia radicans*. *Botanica Marina*, **55**: 435-440.

Gomes-Figueiredo, J., Pimentel, I. C., Vicente, V. A., Pie, M. R., Kava-Cordeiro, V., Galli-Terasawa, L., & Glienke, C. (2007). Bioprospecting highly diverse endophytic *Pestalotiopsis* spp. with antibacterial properties from *Maytenus ilicifolia*, a medicinal plant from Brazil. *Canadian Journal of Microbiology*, **53**: 1123-1132.

Hampl, V., Pavlícek, A., & Flegr, J. (2001). Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with the freeware program FreeTree: application to trichomonad parasites. *International Journal of Systematic and Evolutionary Microbiology*, **51**: 731-735.

Idris, A. M., Al-tahir, I., & Idris, E. (2013). Antibacterial activity of endophytic fungi extracts from the medicinal plant *Kigelia africana*. *Egypt Academic Journal of Biological Sciences*, **5**: 1-9.

Isaka, M., Jaturapat, A., Rukseree, K., Danwisetkanjana, K., Tanticharoen, M., & Thebtaranonth, Y. (2001). Phomoxanthones A and B, novel xanthone dimers from the endophytic fungus *Phomopsis* species. *Journal of Natural Products*, **64**: 1015-1018.

Kanokmedhakul, S., Kanokmedhakul, K., Phonkerd, N., Soytong, K., Kongsaeree, P., & Suksamrarn, A. (2002). Antimycobacterial anthraquinone-chromanone compound and

diketopiperazine alkaloid from the fungus *Chaetomium globosum* KMITL-N0802. *Planta Medica*, **68**: 834-836.

Kusari, S., Pandey, S. P., & Spiteller, M. (2013). Untapped mutualistic paradigms linking host plant and endophytic fungal production of similar bioactive secondary metabolites. *Phytochemistry*, **91**: 81-87.

Lim, C., Kim, J., Choi, J. N., Ponnusamy, K., Jeon, Y., Kim, S. U., & Lee, C. (2010). Identification, fermentation, and bioactivity against *Xanthomonas oryzae* of antimicrobial metabolites isolated from *Phomopsis longicolla* S1B4. *World Journal of Microbiology and Biotechnology*, **20**: 494-500.

Matasyoh, J. C., Dittrich, B., Schueffler, A., & Laatsch, H. (2011). Larvicidal activity of metabolites from the endophytic *Podospora* sp. against the malaria vector *Anopheles gambiae*. *Parasitology Research*, **108**: 561-566.

Momesso, L. D. S., Kawano, C. Y., Ribeiro, P. H., Nomizo, A., Goldman, G. H., & Pupo, M. T. (2008). Chaetoglobosins produced by *Chaetomium globosum*, endophytic fungus found in association with *Viguiera robusta* Gardn (Asteraceae). *Quimica Nova*, **31**: 1680-1685.

Moore, J. H., Davis, N. D., & Diener, U. L. (1972). Mellein and 4-hydroxymellein production by *Aspergillus ochraceus* Wilhelm. *Applied microbiology*, **23**: 1067-1072.

Okanya, P. W., Mohr, K. I., Gerth, K., Jansen, R., & Müller, R. (2011). Marinoquinolines A− F, Pyrroloquinolines from *Ohtaekwangia kribbensis* (Bacteroidetes). *Journal of Natural Products*, **74**: 603-608.

Pelaez, F., Collado, J., Arenal, F., Basilio, A., Cabello, A., Matas, M. D., & Hernández, P. (1998). Endophytic fungi from plants living on gypsum soils as a source of secondary metabolites with antimicrobial activity. *Mycological Research*, **102**: 755-761.

Qiao, M. F., Ji, N. Y., Liu, X. H., Li, K., Zhu, Q. M., & Xue, Q. Z. (2010). Indoloditerpenes from an algicolous isolate of *Aspergillus oryzae*. *Bioorganic & Medicinal Chemistry Letters*, **20**: 5677-5680.

Stadler, M., Wollweber, H., & Fournier, J (2004). A host-specific species of *Hypoxylon* from France, and notes on the chemotaxonomy of The "*Hypoxylon rubiginosum* complex". *Mycotaxon*, **90**: 187–211.

Strobel, G. A., Miller, R. V., Martinez-Miller, C., Condron, M. M., Teplow, D. B., & Hess, W. M. (1999). Cryptocandin, a potent antimycotic from the endophytic fungus *Cryptosporiopsis* cf. *quercina*. *Microbiology*, **145**: 1919-1926.

Tegos, G., Stermitz, F. R., Lomovskaya, O., & Lewis, K. (2002). Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. *Antimicrobial Agents and Chemotherapy*, **46**: 3133-3141.

Tejesvi, M. V., Mahesh, B., Nalini, M. S., Prakash, H. S., Kini, K. R., Subbiah, V., & Shetty, H. S. (2006). Fungal endophyte assemblages from ethnopharmaceutically important medicinal trees. *Canadian Journal of Microbiology*, **52**: 427-435.

Wagenaar, M. M., & Clardy, J. (2001). Dicerandrols, New Antibiotic and Cytotoxic Dimers Produced by the Fungus *Phomopsis longicolla* Isolated from an Endangered Mint. *Journal of Natural Products*, **64**: 1006-1009.

Wang, Q. X., Li, S. F., Zhao, F., Dai, H. Q., Bao, L., Ding, R., & Liu, H. W. (2011). Chemical constituents from endophytic fungus *Fusarium oxysporum*. *Fitoterapia*, **82**: 777-781.

Wang, X. J., Min, C. L., Ge, M., & Zuo, R. H. (2014). An endophytic sanguinarine-producing fungus from *Macleaya cordata*, *Fusarium proliferatum* BLH51. *Current Microbiology*, **68**: 336-341.

Xu, L., Wang, J., Zhao, J., Li, P., Shan, T., Li, X., & Zhou, L. (2010). Beauvericin from the endophytic fungus, *Fusarium redolens*, isolated from *Dioscorea zingiberensis* and its antibacterial activity. *Natural Product Communications*, **5**: 811-814.

Zhang, H. W., Song, Y. C., & Tan, R. X. (2006). Biology and chemistry of endophytes. *Natural Product Reports*, **23**: 753-771.

Zhang, W., Draeger, S., Schulz, B., & Krohn, K. (2009). Ring B aromatic steroids from an endophytic fungus, *Colletotrichum* sp. *Natural Product Communications*, **4**: 1449-1454.

CHAPTER FIVE

DETERMINATION OF ANTIMICROBIAL PHYTOCHEMICALS FROM SELECTED MEDICINAL PLANTS

5.1 Abstract

Phytochemicals occur in plants either as primary or secondary metabolites. They have been known to possess medicinal properties and thus have been used in drug development studies. Emergence and re-emergence of antimicrobial resistance has led researchers back to natural product studies resulting in the discovery of new phytochemical compounds. This study sought to screen leaf extracts of three medicinal plants; *W. ugandensis*, *T. emetica*, and *A. gummifera* for the presence of phytochemicals. Powdered leaf samples were soaked in methanol for 24 h, followed by filtration and evaporation yielding crude methanol fraction. The latter was subjected to column chromatography and thin layer chromatography generating several fractions, which were subjected to phytochemical screening. Phytochemical screening studies revealed the presence of terpenoids, flavonoids, and saponins in all the three plants; phlobatannins were present in *W. ugandensis* and *T. emetica* fraction 2 and absent in *A. gummifera* fractions. Tannins were found to be present in *A. gummifera* but lacking in *W. ugandensis* and *T. emetica*. Results obtained from this study indicate that the three medicinal plants have antimicrobial phytochemicals that can be attributed to their medicinal properties.

5.2 Introduction

Plant-derived compounds have become of great interest in recent times owing to their diverse applications (Bariş *et al*., 2006). Medicinal plants form the richest source of drugs in traditional folk medicine, modern medicine, chemical entities for synthetic drugs, nutraceuticals and food supplements due to the presence of phytochemical constituents (Hammer *et al*., 1999; Nostro *et al*., 2000). Medicinal plants have been used in the treatment of many infections because of their antimicrobial characteristics. Many researchers all over the world have investigated these antimicrobial properties. Interestingly, scientists including, microbiologists, natural product chemists, ethno pharmacologists, and botanists are still carrying out intensive research on phytochemicals from plants, which could later be developed for the treatment of infectious diseases (Tanaka *et al*., 2006). Phytochemicals are secondary metabolites that are

produced by plants with the primary aim of protecting the host against diseases (Ngbede *et al*., 2008). There are two main groups of phytochemicals produced in plants; primary and secondary metabolites. Primary metabolites include chlorophyll, common sugars, and proteins whereas secondary metabolites comprise of alkaloids, anthraquinones, tannins, phenolic compounds, flavonoids, and terpenoids (Krishnaiah *et al*., 2007). Previous studies have demonstrated that these secondary metabolites from medicinal plants are of pharmaceutical value in both human and animal medicine (Kubmarawa *et al*., 2008). Also, secondary metabolites play a significant role in drug development studies. Scientists have focused on studying drugs derived from plant sources as well as other natural sources. This interest is because of the hypothesis that drugs from plant sources are safe and affordable compared to expensive synthetic drugs, which are invariably linked to adverse effects (Bandaranayake et al., 2006), as well as emergence and re-emergence of drug-resistant microorganisms. Antibiotic resistance necessitates the discovery of new harmless and effective pharmacotherapeutic agents from medicinal plants (Rupasinghe *et al*., 2003; Venkataswamy *et al*., 2010). Therefore, this study sought to determine presence of various antimicrobial secondary metabolites in three selected traditional medicinal plants from the Kakamega Tropical Rain Forest.

5.3 Materials and Methods

5.3.1 Sample Collection and Preparation

Fresh leaves of selected medicinal plants; *W. ugandensis*, *T. emetica* and *A. gummifera* were collected from the Kakamega Tropical Rainforest. The three medicinal plants were selected from the 22 plants based on the available information concerning their medicinal properties and use by the local community. The leaves were dried under a shade to prevent evaporation of labile compounds. Thereafter, they were ground into fine powder using Thomas Wiley mill model 4 grinding machine and the powder stored in brown bags for use.

5.3.2 Methanol Extraction

About 500g leaf powder of each plant was soaked in 1.5L of distilled methanol for 24 hours with periodical shaking and filtered through the whatman No.1 filter paper. The filtrate was then concentrated in a vacuum rotavapor at 50°C using Buchi Rotavapor R-205. The methanol crude extracts were placed in the fume hood to total dryness.

5.3.3 Solvent-Solvent Partitioning of Crude Methanol Extract

The crude methanol extracts were divided into two portions where the first portions were subjected to liquid-liquid partitioning by suspending them in water and sequentially extracting with hexane followed by ethyl acetate to acquire three extracts namely; hexane, ethyl acetate and aqueous extracts. The acquired hexane and ethyl acetate extracts were later concentrated using a rotary evaporator at 40°C in order to recover the solvents and residues dried completely in a fume hood. Ethyl acetate extracts were then subjected to extensive thin layer chromatography (TLC) analysis and then column chromatography. The second portions were stored for phytochemical screening.

5.3.4 Thin Layer Chromatography (TLC)

Analytical TLC was performed on silica gel GF 254nm, (Merck, Germany) 0.25mm thickness. Preliminary analysis was performed to identify optimum solvent systems for use as mobile phases. Methanol, ethyl acetate, and chloroform solvent mixtures were tried and modified accordingly, to give optimum separation for ethyl acetate extracts. The solvent mixtures that were giving optimum separations were, 6:4:0.5 methanol-ethyl acetate-water mixtures for *A. gummifera*, 1:1 ethyl acetate-hexane mixtures for *W. ugandensis.* Gradient elution column chromatography was carried out in the case of *T. emetica;* a solvent system of 100-70% chloroform in methanol was used. Visualization was done by illumination under UV lamp (Uvitec- LF-204.LS) at 254 nm and 365 nm.

Samples were spotted on 4×5cm aluminium backed TLC plates. The spotting was done with care being taken not to over load the plate. For each of the different plants, samples were spotted on separate plates. In each plate samples were about half a centimeter from the base and developed to a distance of 4cm in aluminum foil covered TLC. The spotted plates were placed in 100ml glass beakers pre-saturated with the corresponding mobile phase. The developed chromatograms were then visualized under UV light at 254 nm and 365 nm to detect UV visible compounds and determine the profile of the compounds in the respective plant.

5.3.5 Column Chromatography

The optimum solvent systems acquired after an intensive TLC analysis of *W. ugandensis, A. gummifera* and *T. emetica* ethyl acetate crude extracts were prepared to be used in developing column chromatography. Glass column wet packing method was used in which approximately 100g of silica gel were mixed with the respective solvent system into slurry. A padding of cotton

wool was placed at the bottom of the glass column and the slurry mixture allowed settling down at the bottom of the column with gentle tapping to allow proper packing and to get rid of air bubbles. The dry extracts were re-dissolved in a minimum amount of ethyl acetate and separately loaded on evenly packed silica gel columns, by dripping on the column walls, cautioning the disturbance of the silica gel layer. Silica gel 0.06-0.2mm (70-230mesh ASTM) supplied by Scharlau Lab supplies Limited was used for the column chromatography. The columns were eluted gradually with the appropriate mobile phase. The lengths of the columns were 50 cm with a diameter of 20mm and the flow rates were maintained at approximately 15ml/5min. Fractions of equal volumes were collected and further subjected to phytochemical screening.

5.3.6 Phytochemical Screening

Phytochemical tests were done on methanol crude extracts and fractions from column chromatography of the three selected medicinal plants using standard protocol described by Edeoga *et al*. (2005) and Khan *et al*. (2011). Test for tannins was done by adding 0.1% ferric chloride to about 2 ml of the test solution of each extract. The formation of a blue-black coloration indicated the presence of tannins. The test for flavonoids was done by adding about 2 ml of aqueous ammonia solution to about 5 ml of the test solution followed by addition of concentrated H_2SO_4. The appearance of a yellow coloration that disappeared after standing for a while confirmed the presence of flavonoids. Test for terpenoids (Salkowski's test) was done by mixing approximately 5 ml of each extract with 2 ml of $CHCl_3$ and 3 ml of concentrated H_2SO_4 to form a layer. Positive test was indicated by formation of a red coloration on the interface. Test for phlobatannins was done by boiling about 2 ml of the test of each extract with 0.5 ml of 1% HCl, deposition of red precipitate confirmed presence of phlobatannins. Test for saponins was done by adding about 5 ml of distilled water to approximately 5 ml of the test solution followed by adding few drops of olive oil and shaking vigorously. Formation of an emulsion confirmed the presence of saponins.

5.4 Results

Phytochemical screening of the crude methanol leaf extracts and column fractions of ethylacetate extracts revealed the presence of tannins, saponins, phlobatannins, terpenoids, and flavonoids (Table 6). *Warburgia ugandensis* ethylacetate extracts yielded four fractions; *T. emetica* yielded seven fractions whereas *A. gummifera* gave four fractions. These fractions

together with the methanolic crude extracts of each medicinal plant were subjected to phytochemical screening (Table 6). Terpenoids, saponins and flavonoids were found in all the extracts. Tannins on the other hand were present in only methanolic extracts of *A. gummifera* and absent in the rest of the extracts. Phlobatannins were only present in extracts of *W. ugandensis* and *T. emetica.*

5.5 Discussion

In this study, terpenoids, saponins, and flavonoids were present in the leaf extracts of all the three plants. Phytochemical screening of *W. ugandensis* revealed the presence of terpenoids, flavonoids, saponins and phlobatannins. This is in line with studies done by Maobe *et al*. (2013) where *W. ugandensis* leaves extracts constituted of alkaloids, saponins, flavonoids, terpenoids and tannins whereas cardiac glycosides and anthraquinones were absent. Previous studies indicate that *W. ugandensis* comprises of bioactive drimane and colorotane sesquiterpenes (terpenes) which give the plant medicinal properties (Jansen & De Groot, 2004). Phytochemical screening of *T. emetica* showed the presence of flavonoids, terpenoids, phlobatannins and saponins. In agreement with studies that were done in the same plant, phytochemical screening revealed the presence of terpenes, sterols, tannins, flavonoids and phenolics (Nacoulma, 1996). Flavonoid-rich fractions from leaf extracts of *T. emetica* were shown to have effective antioxidant activity (Konaté *et al*., 2014). Phytochemical analysis done on *A. gummifera* in this study showed that the leaf extracts constituted of flavonoids, saponins, terpenoids, and tannins. Previous phytochemical studies have demonstrated that the plants in this genus produce many secondary metabolites that have medicinal value. Such compounds are saponins, terpenes alkaloids and flavonoids (Singab & Bahgat, 2015). For instance, spermine alkaloids were isolated from *A. gummifera* in studies done by Rukunga *et al*. (2007).

Table 6: Results of phytochemical screening done on the selected medicinal plant extracts

Extracts	Phytochemical tested				
	phlob	terp	tan	Sap	flav
Methanol crude extract of *W. ugandensis*	+	+	-	+	+

Methanol crude extract of *A. gummifera*	-	+	+	+	+
Methanol crude extract of *T. emetica*	-	+	-	+	+
W. ugandensis fraction 1	+	+	-	+	+
W. ugandensis fraction 2	+	+	-	+	+
W. ugandensis fraction 3	-	+	-	+	+
W. ugandensis fraction 4	+	+	-	+	+
A. gummifera fraction 1	-	+	-	+	+
A. gummifera fraction 2	-	+	-	+	+
A. gummifera fraction 3	-	+	-	+	+
A. gummifera fraction 4	-	+	-	+	+
Crude ethyl acetate extract *T. emetica*	-	+	-	+	+
T. emetica fraction 1	-	+	-	+	+
T. emetica fraction 2	-	+	-	+	+
T. emetica fraction 3	-	+	-	+	+
T. emetica fraction 4	-	+	-	+	+
T. emetica fraction 5	-	+	-	+	+
T. emetica fraction 6	-	+	-	+	+
T. emetica fraction 7	-	+	-	+	+

Phlob-phlobatannins, terp-terpenoids, tan-tannins, sap-saponins, flav-flavonoids

Other phytochemical studies have also shown that *A. gummifera* extracts constituted of triterpene saponins (Debella *et al*., 2000; Cao *et al*., 2007).

Secondary metabolites occur in plants constitutively; some occur in healthy plants in their biologically active form, whereas others occur as inactive precursors and can only be activated in

response to tissue damage and pathogen attack (Osbourne, 1996). Terpenes are one of the largest classes of secondary metabolites in plants; they include sterols and triterpenes, the latter can accumulate as glycosides in significant quantities in plants. These glycosides are commonly referred to as saponins (Wallace, 2004) and occur widely in plants. They are used as mild detergents and in medicine; they are used as anti-inflammatory, antioxidant and anticancer agents (Malairajan *et al*., 2006) and have been reported to have antifungal activity (Argal, 2006). Phenols, phenolic acid, quinines flavonoids, flavones, tannins, coumarins, and flavonols are classes of secondary metabolites that have been found to exhibit antimicrobial properties. Flavones, flavonoids, and flavanols have been known to be synthesized by plants in response to microbial infection (Bennet & Wallsgrove, 1994). Tannins, which are soluble in water, alcohol and acetone (Basri & Fan, 2005) have been traditionally used in the treatment of wounds, inflamed surfaces of the mouth, diarrhoea, and haemorrhoids.

From this study, it is clear that the three medicinal plants produce different classes of secondary metabolites that have previously been reported to possess antimicrobial activities. Further research should focus on the biological mode of action of these metabolites against disease-causing microorganisms.

References

Argal, A., & Pathak, A. K. (2006). CNS activity of *Calotropis gigantea* roots. *Journal of Ethnopharmacology*, **106**: 142-145.

Bandaranayake, M., A.L. Wickramasinghe, F. Aqil & Owlish, M. (2006). Modern Phytomedicine. Turning Medicinal Plants into Drugs: Quality control, Screening Toxicity and regulation of Herbal Drugs. *WILEY-VCH Versa GmbH and Co.KGaA, WEINNHEIM*, **1**: 25-57.

Bariş, Ö., Güllüce, M., Şahin, F., Özer, H., Kiliç, H., Özkan, H., Sökmen, M., & Özbek, T. (2006). Biological activities of the essential oil and methanol extract of *Achillea biebersteinii* Afan. (Asteraceae). *Turkish Journal of Biology*, **30**: 65-73.

Basri, D. F., & Fan, S. H. (2005). The potential of aqueous and acetone extracts of galls of *Quercus infectoria* as antibacterial agents. *Indian Journal of Pharmacology*, **37**: 26-29

Bennett, R. N., & Wallsgrove, R. M. (1994). Secondary metabolites in plant defence mechanisms. *New Phytologist*, **127**: 617-633.

Cao, S., Norris, A., Miller, J. S., Ratovoson, F., Razafitsalama, J., Andriantsiferana, R., & Kingston, D. G. (2007). Cytotoxic Triterpenoid Saponins of *Albizia gummifera* from the Madagascar Rain Forest. *Journal of Natural Products*, **70**: 361-366.

Debella, A., Haslinger, E., Schmid, M. G., Bucar, F., Michl, G., Abebe, D., & Kunert, O. (2000). Triterpenoid saponins and sapogenin lactones from *Albizia gummifera*. *Phytochemistry*, **53**: 885-892.

Edeoga, H. O., Okwu, D. E., & Mbaebie, B. O. (2005). Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology*, **4**: 685-688.

Hammer, K. A., Carson, C. F., & Riley, T. V. (1999). Antimicrobial activity of essential oils and other plant extracts. *Journal of Applied Microbiology*, **86**: 985-990.

Khan, A. M., Qureshi, R. A., Ullah, F., Gilani, S. A., Nosheen, A., Sahreen, S., Laghari, M.K., Laghari, M.Y., Rehman, S.U., Hussain, I. & Murad, W. (2011). Phytochemical analysis of selected medicinal plants of Margalla Hills and surroundings. *Journal of Medicinal Plant Research*, **5**: 6017-6023.

Konaté, K., Yomalan, K., Sytar, O., Zerbo, P., Brestic, M., Patrick, V. D., & Barro, N. (2014). Free radicals scavenging capacity, antidiabetic and antihypertensive activities of flavonoid-rich fractions from leaves of *Trichilia emetica* and *Opilia amentacea* in an animal model of type 2 diabetes mellitus. *Evidence-Based Complementary and Alternative Medicine*, **2014**: 867075.

Krishnaiah, D., Sarbatly, R., & Bono, A. (2007). Phytochemical antioxidants for health and medicine – A move towards nature. *Biotechnology & Molecular Biology Review*, **1**: 97-104.

Kubmarawa, D., Khan, M. E., Punah, A. M., & Hassan, M. (2008). Phytochemical screening and antibacterial activity of extracts from *Pakia clapperotoniana* keay against human pathogenic bacteria. *Journal of Medicinal Plants Research*, **2**: 352-355.

Malairajan, P., Gopalakrishnan, G., Narasimhan, S., & Veni, K. J. K. (2006). Analgesic activity of some Indian medicinal plants. *Journal of Ethnopharmacology*, **106**: 425-428.

Maobe, M. A., Gatebe, E., Gitu, L., & Rotich, H. (2013). Preliminary phytochemical screening of eight selected medicinal herbs used for the treatment of diabetes, malaria and pneumonia in Kisii region, southwest Kenya. *European Journal of Applied Sciences*, **5**: 01-06.

Nacoulma, O. G. (1996). *Medicinal Plants and their Traditional uses in Burkina Faso*. Doctoral dissertation, Ph. D. Thesis. University of Ouagadougou, Burkina Faso.

Ngbede, J., Yakubu, R. A., & Nyam, D. A. (2008). Phytochemical screening for active compounds in *Canarium schweinfurthii* (Atile) leaves from Jos North, Plateau state, Nigeria. *Research Journal of Biological Sciences,* **3**: 1076-1078.

Nostro, A., Germano, M. P., D'angelo, V., Marino, A., & Cannatelli, M. A. (2000). Extraction methods and bioautography for evaluation of medicinal plant antimicrobial activity. *Letters in Applied Microbiology*, **30**: 379-384.

Osbourn, A. E. (1996). Preformed antimicrobial compounds and plant defense against fungal attack. *The Plant Cell*, **8**:1821-1831.

Rupasinghe, H. V., Jackson, C. J. C., Poysa, V., Di Berardo, C., Bewley, J. D., & Jenkinson, J. (2003). Soyasapogenol A and B distribution in soybean (*Glycine max* L. Merr.) in relation to seed physiology, genetic variability, and growing location. *Journal of Agricultural and Food Chemistry,* **51**: 5888-5894.

Tanaka, J. C. A., Da Silva, C. C., De Oliveira, A. J. B., Nakamura, C. V., & Dias Filho, B. P. (2006). Antibacterial activity of indole alkaloids from *Aspidosperma ramiflorum*. *Brazilian Journal of Medical and Biological Research,* **39**: 387-391.

Venkataswamy, R., Doss, A., Sukumar, M., & Mubarack, H. M. (2010). Preliminary phytochemical screening and antimicrobial studies of *Lantana indica* roxb. *Indian Journal of Pharmaceutical Sciences,* **72**: 229-231.

Wallace, R. J. (2004). Antimicrobial properties of plant secondary metabolites. *Proceedings of the Nutrition Society,* **63**: 621-629.

CHAPTER SIX

GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From this study, it was concluded that endophytic fungi in the genus *Colletotrichum* and *Fusarium* had the highest isolation frequency from leaves of selected medicinal plants, whereas *Aspergillus, Phomopsis, Pestalotiopsis,* and *Cladosporium* had the lowest isolation frequency. The medicinal plant *Clausena anisata* had the highest number of endophytic fungi while,

Afromonum angustifolium, *Vernonia amygdalyna*, and *Barsamia alba* had the least number of endophytic fungi.

Extracts from *Aspergillus* sp. demonstrated antimicrobial activity against test bacteria and fungi at low concentrations. Besides, secondary metabolites produced from *Colletotrichum* sp., *Fusarium* sp., *Chaetomium* sp., *Pestalotiopsis* sp. and *Phomopsis* sp. also showed antimicrobial activity against the test microorganisms.

Phytochemical screening studies of leaf extracts from *W. ugandensis*, *T. emetica* and *A. gummifera* indicated the presence of different classes of phytochemicals including flavanoids, terpenoids, saponins, tannins and phlobatannins. These have previously been reported to have antimicrobial properties against human pathogens.

6.2 Recommendations

1. Further studies should be conducted on alternative methods such as use of β-tubulin gene and Elongation Factor-1 α gene in the identification of fungal endophytes to the species level.
2. Further research should be focused on understanding the chemical nature as well as the *in vivo* biological activities of biomolecules produced by the endophytic fungal species; *Colletotrichum*, *Chaetomium*, *Aspergillus*, *Pestalotiopsis*, *Cladosporium* and *Fusarium*.
3. Biological activities of the phytochemicals identified in this study should be studied against human disease-causing pathogens.

APPENDICES

Appendix 1: Means separation for the activity of the endophytic fungal isolates against *Staphylococcus aureus* and *Pseudomonas aeruginosa in vitro*

	trt	means	M		trt	means	M		trt	means	M
1	POSITIVE.	22.46667	a	23	BA2	12.66667	bcdefgh	45	AG4	9.666667	bcdefgh
2	ML1	17.66667	ab	24	CA4	12.66667	bcdefgh	46	TN2	9.666667	bcdefgh
3	ZG2	16.33333	abc	25	TA2	12.66667	bcdefgh	47	TI1	9.333333	bcdefghi
4	MW2	16	abcd	26	PF2	12.33333	bcdefgh	48	CM2	9	bcdefghi
5	PC4	16	abcd	27	SB1	12.33333	bcdefgh	49	PA4	9	bcdefghi
6	BS2	15.66667	abcde	28	VA1	12.33333	bcdefgh	50	TE3	8.666667	bcdefghi
7	CM3	15.66667	abcde	29	CA2	11.66667	bcdefgh	51	ML2	8.333333	bcdefghi
8	PA5	15.66667	abcde	30	ML4	11.66667	bcdefgh	52	PA1	8.333333	bcdefghi
9	PC3	15.66667	abcde	31	PA2	11.66667	bcdefgh	53	PF1	8	cdefghi
10	TD1	15.66667	abcde	32	TE1	11.66667	bcdefgh	54	PA3	6.666667	defghi
11	MW4	15.33333	abcde	33	ZG4	11.33333	bcdefgh	55	AG2	6.333333	efghi
12	PC1	15	abcdef	34	CA3	11	bcdefgh	56	CA1	5.666667	fghi
13	BA1	14.66667	abcdefg	35	MW3	11	bcdefgh	57	TN3	5.666667	fghi
14	PC2	14.66667	abcdefg	36	WU1	11	bcdefgh	58	CM1	5.333333	ghi
15	PC5	14.66667	abcdefg	37	WU4	11	bcdefgh	59	KA2	4.666667	hi
16	TD2	14.33333	abcdefg	38	ML3	10.66667	bcdefgh	60	BS1	0	i
17	KA1	14	abcdefgh	39	RC2	10.33333	bcdefgh	61	MW1	0	i
18	TA1	14	abcdefgh	40	TE4	10.33333	bcdefgh				
19	TN1	13.66667	abcdefgh	41	AA1	10	bcdefgh				
20	TE2	13	bcdefgh	42	ES1	10	bcdefgh				
21	WU2	13	bcdefgh	43	RC1	10	bcdefgh				
22	WU3	13	bcdefgh	44	ZG3	10	bcdefgh				

	trt	means	M		trt	means	M		trt	means	M
1	POSITIVE.CONTROL	19.66667	a	23	CA1	14	abcdefgh	45	CM.1	9.666667	efghijk
2	PC5	17	ab	24	CA3	14	abcdefgh	46	PF2	9.666667	efghijk
3	RC1	17	ab	25	PA2	14	abcdefgh	47	ML3	9.333333	fghijk
4	BA1	16.66667	ab	26	TN2	14	abcdefgh	48	TE2	9.333333	fghijk
5	BS2	16.66667	ab	27	ES1	13.66667	bcdefghi	49	MW2	8.666667	ghijk
6	CM3	16.33333	abc	28	MW4	13.66667	bcdefghi	50	ZG1	8.666667	ghijk
7	SB1	16.33333	abc	29	TA1	13.66667	bcdefghi	51	ZG4	8.333333	hijk
8	TN3	16.33333	abc	30	CA2	13.33333	bcdefghij	52	KA2	8	ijk
9	WU1	16.33333	abc	31	KA1	13	bcdefghijk	53	AG2	7.666667	jk
10	CM2	15.66667	abcd	32	ML2	13	bcdefghijk	54	TD2	7.666667	jk
11	PA1	15.66667	abcd	33	PC3	13	bcdefghijk	55	TE1	7.333333	k
12	AA	15.33333	abcde	34	PF1	13	bcdefghijk	56	VA	7.333333	k
13	BA2	15.33333	abcde	35	CA4	12.66667	bcdefghijk	57	MW1	0	l
14	PC1	15.33333	abcde	36	TE3	11.66667	bcdefghijk	58	TE4	0	l
15	TI1	15.33333	abcde	37	ML1	11.33333	bcdefghijk	59	WU2	0	l
16	AG4	15	abcdef	38	MW3	11.33333	bcdefghijk	60	WU3	0	l
17	ML4	14.66667	abcdef	39	PC4	10.66667	cdefghijk	61	WU4	0	l
18	PC2	14.66667	abcdef	40	ZG2	10.66667	cdefghijk				
19	TA2	14.66667	abcdef	41	RC2	10.33333	defghijk				
20	BS1	14.33333	abcdefg	42	TD1	10.33333	defghijk				
21	PA4	14.33333	abcdefg	43	TN1	10.33333	defghijk				
22	PA5	14.33333	abcdefg	44	PA3	10	defghijk				

Trt- Treatments; M-levels of significance

Appendix 2: ANOVA summary output of endophytic fungal isolates against *S. aureus* and *P. aeruginosa in vitro*

```
> summary(anova_results)
             Df Sum Sq Mean Sq F value Pr(>F)
ind          60   2847   47.46   6.225 <2e-16 ***
Residuals   122    930    7.62
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
```

```
> summary(anova_results)
             Df Sum Sq Mean Sq F value Pr(>F)
ind          60   2847   47.46   6.225 <2e-16 ***
Residuals   122    930    7.62
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
```

Appendix 3: ITS consensus sequences of endophytic fungal isolates

S/N	Endophyte isolate code	Sequence	Species	Identity %
1	PF1	TGACCGCGGAGGTACCGAGTTATACA ACTCATCAACCCTGTGAACATACCTAAAACGTTGCTTCGGCGGGAACAGA CGGCCCTGTAACAACGGGCCGCCCCCGCCAGAGGACCCCTAACTCTGTTT TTATAATGTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAA CGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATT GCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACC CTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGTGGGC ACACGCCGTCCCTCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCG TAGTAGCTAACACCTCGCAACTGGAGAGCGGCGCGGCCATGCCGTAAAAC ACCCAACTTCTGAATGTTGACCTCGAATCAGGTAGGAATACCCGCTGAAC TTAAGCATA	*Fusarium*	100
2	PC1	GCGGANGGATCATTACCGAGTTTAC NACTCCCAAACCCCTGTGAACATACCACTTGTTGCCTCGGCGGATCAGCC CGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTT CTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAA CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAG TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATT GCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACC CTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAAT TGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTT ACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGA CCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCAT	*Fusarium*	99
3	TN2	GGCGGANGGACTACTGAGTTACCGCT CATAACCCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGTAGACGG CCTCGTGAAACGGGCCTCTCCCGGCCGCCCGTCCGCGGGTGGCGCCCGCC GGAGGATAACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAG TATAATAATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATG AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCAT GCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCC CTACGGTCGACGTAGGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCT CCTTTGCGTAGTAACATTTCGTCTCGCACTGGGATCCGGAGGGACTCTTG CCGTAAAACCCCCAATTTTTCTAAAGGTTGACCTCGGATCAGGTAGGAAT ACCCGCTGAACTTAAGCATATCNAAAA	*Colletotrichum*	99
4	TN3	GGGACCGCGGAGGGCATTACCGAGTTTACACT CCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCGGATCAGCCCGCT CCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTAT ATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGA TCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAAT GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC CCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCA AGCCCAGCTTGGTGTTGGGACTCGCGAGTCAAATCGCGTTCCCCAAATTG ATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTAC TGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACC TCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATA	*Fusarium*	99
5	PC2	GAGTTTACGCTCTAC AACCCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGTGGCGGCCTC GCGGCCGCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGAGGTCCACTA AACTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAATCAAA ACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTT GAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCG TCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTACGGCTGACGT AGGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTA ACTTAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCC CAATTTTTACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTT AAGCATAA	*Colletotrichum*	99
6	TN1	GGGCGGAGGGTACCGAGTTTACNACT CCCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATCAGCCCGC GCCCGGTAAAACGGGACGGCCCGCCCGAGGACCCTAAACTCTGTTTTTAG TGGAACTTCTGAGTAAAACAAACAAATAAATCAAAACTTTCAACAACGGA TCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAAT GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC	*Fusarium*	99

		CCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCA AGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGAT TGGCGGTCACGTCGAGCTTCCATAGCGTAGTAATCATACACCTCGTTACT GGTAATCGTCGCGGCCACGCCGTAAAACCCCAACTTCTGAATGTTGACCT CGGATCAGGTAGGAATACCCGCTGAACTTAAGCAT		
7	TI1	GACGGAGGGTACTGAGTTACCGCT CTATAACCCTTTGTGAACATACCTACAACTGTTGCTTCGGCGGGTAGGCC GTCCCCTGAAAAGGACGCCTCCCGGCCCGGACCGGACCCCCTGTGGGGCC GGACTCGGCGCCCGCCGGAGGATAACCAAACGCTATTTTAACGACGTTTC TTCTGAGTGGCATAAGCAAAATAATCAAAACTTTTAACAACGGATCTCTT GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCA GCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCT GCTTGGTGTTGGGGCTCTACGGTCGACGTAGGCCCTCAAAGGTAGTGGCG GACCCTCCCGGAGCCTCCTTTGCGTAGTAACATTTCGTCTCGCACTGGGA TCCGGAGGGACTCTTGCCGTAAAACCCCCCAATTTTTCAAGGTTGACCTC GGATCAGGTAGGAATACCCGCTGAACTTAAGCGAA	*Colletotrichum*	100
8	TA2	CGGGCGGAGATTACTGAGTTTACGCT CTACAACCCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGTGGCGG CCTCGCGGCCGCCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGAGGTCC ACTAAACTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAAT CAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT CTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCG AGCGTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTACGGCTG ACGTAGGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGT AGTAACTTAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAAC CCCCCAATTTTTACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGA ACTTAAGCATA	*Colletotrichum*	99
9	TI3	CTTACTGAGTTTACGC TCTACAACCCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGTGGCG GCCTCGCGGCCGCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGAGGTC CACTAAACTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAA TCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC AGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA TCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTC GAGCGTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTACGGCT GACGTAGGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCG TAGTAACTTAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAA CCCCCCAATTTTTACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTG AACTTAAGCATATCAATAAGGAGGAA	*Colletotrichum*	99
10	TI2	TGGGCTTACTGAGTTTACGCT CTACAACCCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGTGGCGG CCTCGCGGCCGCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGAGGTCC ACTAAACTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAAT CAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT CTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCG AGCGTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTACGGCTG ACGTAGGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGT AGTAACTTAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAAC CCCCCAATTTTTACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGA ACTTAAGCA	*Colletotrichum*	99
11	PF2	TGGGCNTACCGAGTTTACAAC TCCCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATCAGCCCG CGCCCGGTAAAACGGGACGGCCCGCCCGAGGACCCTAAACTCTGTTTTTTA GTGGAACTTCTGAGTAAAACAAACAAATAAATCAAAACTTTCAACAACGG ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAA TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTC AAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGA TTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAATCATACACCTCGTTAC TGGTAATCGTCGCGGCCACGCCGTAAAACCCCAACTTCTGAATGTTGACC TCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATA	*Fusarium*	100
12	WU1	CGGTGACCGCGGAGTTACTGAGTTTACGCTCTA CAACCCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGTGGCGGCCT CGCGGCCGCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGAGGTCCACT AAACTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAATCAA AACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCG AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT	*Colletotrichum*	99

		TGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGC GTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTACGGCTGACG TAGGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGT AACTTAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCC CCAATTTTTACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACT TAAGCAT		
13	AA1	CGACCGAGTTATACAACTCATCAA CCCTGTGAACATACCTAAAACGTTGCTTCGGCGGGAACAGACGGCCCTGT AACAACGGGCCGCCCCCGCCAGAGGACCCCTAACTCTGTTTTTATAATGT TTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCT TGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCC AGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCC CCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGTGGGCACACGCCGT CCCTCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGCTA ACACCTCGCAACTGGAGAGCGGCGCGGCCATGCCGTAAAACACCCAACTT CTGAATGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGCATA TCAATAACGGAG	*Fusarium*	99
14	KA1	GGCGGAGGGTACTGAGTTACCGCTCTA TAACCCTTTGTGAACATACCTACAACTGTTGCTTCGGCGGGTAGGCCGTC CCCTGAAAAGGACGCCTCCCGGCCCGGACCGGACCCCCTGTGGGGCCGGA CTCGGCGCCCGCCGGAGGATAACCAAACGCTATTTTAACGACGTTTCTTC TGAGTGGCATAAGCAAAATAATCAAAACTTTTAACAACGGATCTCTTGGT TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCA TTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCT TGGTGTTGGGGCTCTACGGTCGACGTAGGCCCTCAAAGGTAGTGGCGGAC CCTCCCGGAGCCTCCTTTGCGTAGTAACATTTCGTCTCGCACTGGGATCC GGAGGGACTCTTGCCGTAAAACCCCCCAATTTTTCAAGGTTGACCTCGGA TCAGGTAGGAATACCCGCTGAACTTAAGCATGGAAAA	*Colletotrichum*	100
15	WU3	GGCGGATTACTGAGTTTACGCTCTA CAACCCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGTGGCGGCCT CGCGGCCGCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGAGGTCCACT AAACTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAATCAA AACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCG AAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTT TGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGC GTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTACGGCTGACG TAGGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGT AACTTAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCC CCAATTTTTACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACT TAAGCATGGGAAAAA	*Colletotrichum*	99
16	ES1	GGGACCGCGGAGTTACCGAGTTTACACTCCC AAACCCCTGTGAACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCC GGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATG TAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCT CTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCG CCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGC ACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTCAAATTGATTGG CGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGTA ATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGGA TCAGGTAGGAATACCCGCTGAACTTAAGCAT	*Fusarium*	100
17	PC5	TAGGCATTACTGAGTTT ACGCTCTACAACCCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGT GGCGGCCTCGCGGCCGCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGA GGTCCACTAAACTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAA ATAATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGA ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT CGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCT GTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTAC GGCTGACGTAGGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTT TGCGTAGTAACTTAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGT AAAACCCCCCAATTTTTACAAGGTTGACCTCGGATCAGGTAGGAATACCC GCTGAACTTAAGCATATCAATAAGGAGGAA	*Colletotrichum*	99
18	TE2	TGAGGTTGCTGGAACGCGCCCCTGGCG CACCCAGAAACCCTTTGTGAACTTATACCTTACTGTTGCCTCGGCGCAGG CCGTCCCCCCTGGGGTCCCTTGGAGACAAGGAGCAGCCGGCCGGTGGCCA	*Phomopsis*	99

		AATTAACTCTGTTTTTACACTGAAACTCTGAGTACAAAACATAAATGAAT CAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT CTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCG AGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGATGGGGCACTGCTTTTT CCGAAAAGCAGGCCCTGAAATATAGTGGCGAGCTCGCCAGGACTCCGAGC GTAGTAGTTAAACCCTCGCTTTGGAAGGCCTGGCGGTGCCCTGCCGTTAA ACCCCAACTTTTGAAAATTTGACCTCGGATCAGGTAGGAATACCCGCTGA ACTTAAGC		
19	AG2	GCGGGGATTACTGAGTGAGGGTCCCTCGGG GCCCAACCTCCCACCCGTGTATACCGTACCTTGTTGCTTCGGCGAGCCCG CCCCCCTTTCCTTAGGGGTGGCACAGCGCTCGCCGGAGACACCAACGTGA ACACTGTCTGAAGTTTTGTCGTCTGAGTCGATTGTATCGCAATCAGTTAA AACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCG AAATGCGATAATTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTT TGAACGCACATTGCACCCCCTGGTATTCCGGGGGGTATGCCTGTCCGAGC GTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGTCGTCGTCCCCCCC GGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCG TATGGGGCTTTGTCACCCGCTCTTGTAGGCCCGGCCGGCTGCTGGCCGAC GCTGAAAAGCAACCAATCTATTTCATCAGGTTGACCTCGGATCAGGTAGG GATACCCGCTGAACTTAAGGGA	*Aspergillus*	99
20	PC4	GTTACTGAGTTTACGCTCTACA ACCCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGTGGCGGCCTCG CGGCCGCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGAGGTCCACTAA ACTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAATCAAAA CTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG AACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGT CATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTACGGCTGACGTA GGCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAA CTTAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCC AATTTTTACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTA AGCA	*Colletotrichum*	99
21	KA2	GGCATTGCTGGAACGCGCTTCGG CGCACCCAGAAACCCTTTGTGAACTTATACCTTTTAGTTGCCTCGGCGTC AGGCCGGCCCCCTAGGGGCCCCTCGGAGACGAGGAGCAGGCCCGCCGACG GTATATCAAACTCTTGTTTTTACACTGAAACTCTGAGAAATAAACATAAA TGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGA ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT CGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCT GTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGATGGGGCACTGC TTCGCAAGGAGCAGGCCCTGAAATCTAGTGGCGAGCTCGCCAGGACCCCG AGCGCAGTAGTTAAACCCTCGCTTTGGAAGGCCCTGGCGGTGCCCTGCCG TTAAACCCCCAACTTTTGAAAATTTGACCTCGGATCAGGTAGGAATACCC GCTGAACTTAAGCATATGGAG	*Phomopsis*	98
22	WU2	CCGTGAACGCGGGGGCATTACCGAGT TTACNACTCCCAAACCCCTGTGAACATACCACTTGTTGCCTCGGCGGATC AGCCCGCTCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCT GTTTCTATATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCA ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGA TAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA CATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTC AACCCTCAAGCACAGCTTGGTGTTGGGACTCGCGTTAATTCGCGTTCCTC AAATTGATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCT CGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATG TTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATA	*Fusarium*	99
23	TE1	GCGGGCTACAGAGTTGCAAAACTCCC ACAAACCATCGCGAATCTTACCCGTACGGTTGCCTCGGCGCTGGCGGTCC GGAAAGGCCTTCGGGCCCTCCCGGATCCTCGGGTCTCCCGCTCGCGGGAG GCTGCCCGCCGGAGTGCCGAAACTAAACTCTTGATATTTTATGTCTCTCT GAGTAAACTTTTAAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTC TGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGTATT CTGGCGAGCATGCCTGTTCGAGCGTCATTTCAACCATCAAGCTCTGCTTG CGTTGGGGATCCGCGGCTGTCCGCGGTCCCTCAAAATCAGTGGCGGGCTC GCTAGTCACACCGAGCGTAGTAACTCTACATCGCTATGGTCGTGCGGCGG GTGCTTGCCGTAAAACCCCCCATTTCTAAGGTTGACCTCGGATCAGGTAG GAATACCCGCTGAACTTAAGGG	*Chaetomium*	100

24	TD2	GACCCGGAGTTATAGAGTTTTCTAAACT CCCAACCCATGTGAACTTACCATTGTTGCCTCGGCAGAAGCTGCTCGGTG CACCCTACCTTGGAACGGCCTACCCTGTAGCGCCTTACCCTGGAACGGCT TACCCTGTAACGGCTGCCGGTGGACTACCAAACTCTTGTTATTTTATTGT AATCTGAGCGTCTTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCT TGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATT AGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCT AGCTTAGTGTTGGGAGCCTACTGCTTTTGCTAGCTGTAGCTCCTGAAATA CAACGGCGGATCTGCGATATCCTCTGAGCGTAGTAAATTTTTATCTCGCT TTTGACTGGAGTTGCAGCGTCTTTGGCCGCTAAATCCCCCAATTTTTAAT GGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCAT	*Pestalotiopsis*	100
25	TD1	TCGGAGGCATTATAGA GTTTTCTAAACTCCCAACCCATGTGAACTTACCATTGTTGCCTCGGCAGA AGCTGCTCGGTGCACCCTACCTTGGAACGGCCTACCCTGTAGCGCCTTAC CCTGGAACGGCTTACCCTGTAACGGCTGCCGGTGGACTACCAAACTCTTG TTATTTTATTGTAATCTGAGCGTCTTATTTTAATAAGTCAAAACTTTCAA CAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC ATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCA ACCCTTAAGCCTAGCTTAGTGTTGGGAGCCTACTGCTTTTGCTAGCTGTA GCTCCTGAAATACAACGGCGGATCTGCGATATCCTCTGAGCGTAGTAAAT TTTTATCTCGCTTTTGACTGGAGTTGCAGCGTCTTTGGCCGCTAAATCCC CCAATTTTTAATGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTT AAGCATATGAAGGAA	*Pestalotiopsis*	100
26	AG1	ACTGAGTTACGCTCTACAACCC TTTGTGAACATACCTTTAANTGTTGCTTCGGCGGGTGGCGGCCTCGCGGC CGCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGAGGTCCACTAAACTC TGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAATCAAAACTTT TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG CACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATT TCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTACGGCTGACGTAGGCC CTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACTTA ACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCCAATT TTTACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCA TATCAATAAGCGGAGGAA	*Colletotrichum*	99
27	BA2	GACGGGCATTATAGAGTTTTCT AAACTCCCAACCCATGTGAACTTACCTTTTGTTGCCTCGGCAGAAGTTAT AGGTCTTCTTATAGCTGCTGCCGGTGGACCATTAAACTCTTGTTATTTTA TGTAATCTGAGCGTCTTATTTTAATAAGTCAAAACTTTCAACAACGGATC TCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCC ATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAG CCTAGCTTAGTGTTGGGAATCTACTTCTCTTAGGAGTTGTAGTTCCTGAA ATACAACGGCGGATTTGTAGTATCCTCTGAGCGTAGTAATTTTTTTCTCG CTTTTGTTAGGTGCTATAACTCCCAGCCGCTAAACCCCCAATTTTTTGTG GTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATA	*Pestalotiopsis*	100
28	CA4	GGCGGAGTTACCGAGTTATACAACTC ATCAACCCTGTGAACATACCTAAAACGTTGCTTCGGCGGGAACAGACGGC CCTGTAACAACGGGCCGCCCCCGCCAGAGGACCCCTAACTCTGTTTTTAT AATGTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGA TCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC CCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTCA GGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGTGGGCACAC GCCGTCCCTCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGT AGCTAACACCTCGCAACTGGAGAGCGGCGCGGCCATGCCGTAAAACACCC AACTTCTGAATGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAA GCA	*Fusarium*	100
29	VA1	GAAGGGTTACCGAGTTTACAACTCCCA AACCCCTGTGAACATACCAATTGTTGCCTCGGCGGATCAGCCCGCTCCCG GTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGT AACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGGATCTC TTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGA ATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGC CAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCC CAGCTTGGTGTTGGGACTCGCGAGTCAAATCGCGTTCCCCAAATTGATTG GCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTACTGGT AATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGACCTCGG	*Fusarium*	100

		ATCAGGTAGGAATACCCGCTGAACTTAAGCATATCGGGAGGA		
30	CA3	CCGGATTACTGAGTTTACGCTCTACAA CCCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGTGGCGGCCTCGC GGCCGCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGAGGTCCACTAAA CTCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAATCAAAAC TTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA ACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTC ATTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTACGGCTGACGTAG GCCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAAC TTAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCCA ATTTTTACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAA GCATATCAATAACGGAG	*Colletotrichum*	99
31	CA2	GGAGTTACTGAGTTTACGCT CTACAACCCTTTGTGAACATACCTATAACTGTTGCTTCGGCGGGCAGGGT CTCCGTGACCCTCCCGGCCTCCCGCCCCCGGGCGGGTCGGCGCCCGCCGG AGGATAACCAAACTCTGATTTAACGACGTTTCTTCTGAGTGGTACAAGCA AATAATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAG AACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA TCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCC TGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGCCCTA CGGCTGACGTAGGCCCTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCT TTGCGTAGTAACTTTACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCG TAAAACCCCCAATTTTCCAAAGGTTGACCTCGGATCAGGTAGGAATACCC GCTGAACTTAAGCATATGCGGAGGAA	*Colletotrichum*	99
32	CA1	GGGACCGCGGAGTTACTGAGTTACCGCTCTA TAACCCTTTGTGAACATACCTACAACGTTGCCTCGGCGGGCAGCCGGAGC CCAGCTCCGGCGCCCGGAGCCGCCGTCTCGGCGCGCCCCACCCGCCGGGG GATTACCAAACTCTATTTTAACGACGTCTCTTCTGAGTGGCACAAGCAAA TAATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATC GAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTG TTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGCGTTGGGGCCCTACG GCTGACGTAGGCCCCGAAATACAGTGGCGGACCCTCCCGGAGCCTCCTTT GCGTAGTAACATACCACCTCGCACTGGGATCCGGAGGGACTCCTGCCGTA AAACCCCCCAATTTTTCAAGGTTGACCTCGGATCAGGTAGGAATACCCGC TGAACTTAAGCATA	*Colletotrichum*	98
33	ML3	AACCGCGGAGTTACCGAGTTATACAACTCATC AACCCTGTGAACATACCTAAAACGTTGCTTCGGCGGGAACAGACGGCCCT GTAACAACGGGCCGCCCCCGCCAGAGGACCCCTAACTCTGTTTTTATAAT GTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCT CTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCG CCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGC CCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGTGGGCACACGCC GTCCCTCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTAGTAGC TAACACCTCGCAACTGGAGAGCGGCGCGGCCATGCCGTAAAACACCCAAC TTCTGAATGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTTAAGCA TATCGGAG	*Fusarium*	100
34	ML2	GACCGCGGAGGTTACCGAGTTTACAAC TCCCAAACCCCTGTGAACATACCAATTGTTGCCTCGGCGGATCAGCCCGC TCCCGGTAAAACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTA TATGTAACTTCTGAGTAAAACCATAAATAAATCAAAACTTTCAACAACGG ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAA TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG CCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTC AAGCCCAGCTTGGTGTTGGGACTCGCGAGTCAAATCGCGTTCCCCAAATT GATTGGCGGTCACGTCGAGCTTCCATAGCGTAGTAGTAAAACCCTCGTTA CTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAATGTTGAC CTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATGG AGGA	*Fusarium*	100
35	TA1	CGGGTACTGAGTTTACGCTCTACAAC CCTTTGTGAACATACCTTTAACTGTTGCTTCGGCGGGTGGCGGCCTCGCG GCCGCCCCCGGCCCCGCTCACGCGGGGCGCCCGCCGGAGGTCCACTAAAC TCTGATTTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAATCAAAACT TTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAAT GCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA CGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCA TTTCAACCCTCAAGCACCGCTTGGTTTTGGGGCCCTACGGCTGACGTAGG CCCTTAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACT	*Colletotrichum*	99

		TAACGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCCAA TTTTTACAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAG CATA		
36	ML4	AGCGGAGGTTACCGAGTTATACAAC TCATCAACCCTGTGAACATACCTAAAACGTTGCTTCGGCGGGAACAGACG GCCCTGTAACAACGGGCCGCCCCCGCCAGAGGACCCCTAACTCTGTTTTT ATAATGTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACG GATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTA ATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGC GCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCT CAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGTGGGCAC ACGCCGTCCCTCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGTA GTAGCTAACACCTCGCAACTGGAGAGCGGCGCGGCCATGCCGTAAAACAC CCAACTTCTGAATGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACTT AAGCAT	*Fusarium*	100
37	TE4	GGAGTACTGAGTTACC GCTCTATAACCCTTTGTGAACATACCTAACCGTTGCTTCGGCGGGCAGGG GAAGCCTCTCGCGGGCCTCCCCTCCCGGCGCCGGCCCCCACCACGGGGAC GGGGCGCCCGCCGGAGGAAACCAAACTCTATTTACACGACGTCTCTTCTG AGTGGCACAAGCAAATAATTAAAACTTTTAACAACGGATCTCTTGGTTCT GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCATTC TGGCGAGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGG TTTTGGGGCCCCACGGCACACGTGGGCCCTTAAAGGTAGTGGCGGACCCT CCCGGAGCCTCCTTTGCGTAGTAACTAACGTCTCGCACTGGGATTCGGAG GGACTCTTGCCGTAAAACCCCCAAATTTTTTACAGGTTGACCTCGGATCA GGTAGGAATACCCGCTGAACTTAAGCATAT	*Colletotrichum*	100
38	TE5	GGTGACGCGGAGTGCTGGAACGCGCC CCTGGCGCACCCAGAAACCCTTTGTGAACTTATACCTTACTGTTGCCTCG GCGCAGGCCGTCCCCCCTGGGGTCCCTTGGAGACAAGGAGCAGCCGGCCG GTGGCCAAATTAACTCTGTTTTTACACTGAAACTCTGAGTACAAAACATA AATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC ATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGC CTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGATGGGGCACT GCTTTTTCCGAAAAGCAGGCCCTGAAATATAGTGGCGAGCTCGCCAGGAC TCCGAGCGTAGTAGTTAAACCCTCGCTTTGGAAGGCCTGGCGGTGCCCTG CCGTTAAACCCCAACTTTTGAAAATTTGACCTCGGATCAGGTAGGAATAC CCGCTGAACTTAAGCATATGGGAGGAA	*Phomopsis*	99
39	WU5	GGGGCATTATCGAGTTACCACTCTATAA CCCTTTGTGAACATACCTACATGTTGCTTCGGCGGTCGGCCCCCCGGGCC CCCGGCCCCGCTCACGCGGGGCGTCCGCCGGAGGATAACCAAACTCTGAT TTAACGACGTTTCTTCTGAGTGGCACAAGCAAATAATCAAAACTTTTAAC AACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATA AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACA TTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAA CCCTCAAGCACTGCTTGGTGTTGGGGCTCTACGGTTGACGTAGGCCCCCA AAACTAGTGGCGGACCCTCTCGGAGCCTCCTTTGCGTAGTAACTTTTGTC TCGCACTGGGATTCGGAGGGATTCTAGCCGTTAAACCCCCAATTTTCTAA AGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATC ACGGAGGA	*Colletotrichum*	100
40	ZG1	GCGGAGGGTTACTGAGTTACCGCTCT ATAACCCTTTGTGAACATACCTAACCGTTGCTTCGGCGGGCAGGGGAAGC CTCTCGCGGGCCTCCCCTCCCGGCGCCGGCCCCAACCACGGGGACGGGGC GCCCGCCGGAGGAAACCAAACTCTATTTACACGACGTCTCTTCTGAGTGG CACAAGCAAATAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCAT CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA GTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCATTCTGGCG AGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGTTTTG GGGCCCCACGGCACACGTGGGCCCTTAAAGGTAGTGGCGGACCCTCCCGG AGCCTCCTTTGCGTAGTAACTAACGTCTCGCACTGGGATTCGGAGGGACT CTTGCCGTAAAACCCCCAAATTTTTTTACAGGTTGACCTCGGATCAGGTA GGAATACCCGCTGAACTTAAGCAT		
41	RC1	GGCATTACTGAGTT ACCGCTCTATAACCCTTTGTGAACATACCTAACCGTTGCTTCGGCGGGCA GGGGAAGCCTCTCGCGGGCCTCCCCTCCCGGCGCCGGCCCCCACCACGGG GACGGGGCGCCCGCCGGAGGAAACCAAACTCTATTTACACGACGTCTCTT CTGAGTGGCACAAGCAAATAATTAAAACTTTTAACAACGGATCTCTTGGT	*Colletotrichum*	100

		TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCA TTCTGGCGAGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCT TGGTTTTGGGGCCCCACGGCACACGTGGGCCCTTAAAGGTAGTGGCGGAC CCTCCCGGAGCCTCCTTTGCGTAGTAACTAACGTCTCGCACTGGGATTCG GAGGGACTCTTGCCGTAAAACCCCCAAATTTTTTACAGGTTGACCTCGGA TCAGGTAGGAATACCCGCTGAACTTAAGCATATGGAGA		
42	AG3	GCTTACCGAGTTATACAA CTCATCAACCCTGTGAACATACCTAAAACGTTGCTTCGGCGGGAACAGAC GGCCCTGTAACAACGGGCCGCCCCCGCCAGAGGACCCCTAACTCTGTTTT TATAATGTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAAC GGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTG CGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCC TCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTGTGGGCA CACGCCGTCCCTCAAATACAGTGGCGGTCCCGCCGCAGCTTCCATTGCGT AGTAGCTAACACCTCGCAACTGGAGAGCGGCGCGGCCATGCCGTAAAACA CCCAACTTCTGAATGTTGACCTCGAATCAGGTAGGAATACCCGCTGAACT TAAGCATAAA	*Fusarium*	100
43	RC2	GTGAGCGGCATTACAAGTGACCCCG GTCTAACCACCGGGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCT CCGGGGCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACT CTTGCGTAACTTTGCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTT TAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG CACATTGCGCCCCCTGGTATTCCGGGGGGGCATGCCTGTTCGAGCGTCATT TCACCACTCAAGCCTCGCTTGGTATTGGGCAACGCGGTCCGCCGCGTGCC TCAAATCGACCGGCTGGGTCTTCTGTCCCCTAAGCGTTGTGGAAACTATT CGCTAAAGGGTGTTCGGGAGGCTACGCCGTAAAACAACCCCATTTCTAAG GTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCAT	*Cladosporium*	100
44	RC1b	GAGCGGAGGGCATTACTGAGTTACCGCTC TATAACCCTTTGTGAACATACCTAACCGTTGCTTCGGCGGGCAGGGGAAG CCTCTCGCGGGCCTCCCCTCCCGGCGCCGGCCCCCACCACGGGGACGGGG CGCCCGCCGGAGGAAACCAAACTCTATTTACACGACGTCTCTTCTGAGTG GCACAAGCAAATAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCA TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC AGTGAATCATCGAATCTTTGAACGCACATTGCGCTCGCCAGCATTCTGGC GAGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGTTTT GGGGCCCCACGGCACACGTGGGCCCTTAAAGGTAGTGGCGGACCCTCCCG GAGCCTCCTTTGCGTAGTAACTAACGTCTCGCACTGGGATTCGGAGGGAC TCTTGCCGTAAAACCCCCAAATTTTTTACAGGTTGACCTCGGATCAGGTA GGAATACCCGCTGAACTTAAGCAT	*Colletotrichum*	99
45	MW4	GGCGGAGGACATTACCGAGTT ATACAACTCATCAACCCTGTGAACATACCTAANACGTTGCTTCGGCGGGA ACAGACGGCCCTGTAACAACGGGCCGCCCCCGCCAGAGGACCCCTAACTC TGTTTTTATAATGTTTTTCTGAGTAAACAAGCAAATAAATTAAAACTTTC AACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCG ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC ACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTA CAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAAGCCCCCTG TGGGCACACGCCGTCCCTCAAATACAGTGGCGGTCCCGCCGCAGCTTCCA TTGCGTAGTAGCTAACACCTCGCAACTGGAGAGCGGCGCGGCCATGCCGT AAAACACCCAACTTCTGAATGTTGACCTCGAATCAGGTAGGAATACCCGC TGAACTTAAGCATATCGGAGGA	*Fusarium*	99
46	MW3	GGTGACCTGCGGAGGCATTACCGAGAGTTGTAG GCTTCTGTCTACCATCTCTTACCCATGTCTTTTGCGTACTACACGTTTCC TCGGCAGGTCCGCCTGCCGTTAGGACAATTTAAACCATTTGCAGTTGCAG TCAGCGTCTGAAAAACTTAATAATTACAACTTTTAACAACGGATCTCTTG GTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATT GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGG TATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCAAGCTCTG CTTGGTGTTGGGTGTTTGTCCCGCCTCTGCGCGTGGACTCGCCTTAAAGC AATTGGCAGCCGACGTATTGTTTCGGAGCGCAGCACAAATTGCGCTTTGT AGCTACTACGACAGCATCCATCAAGCCTTTTTTCACGCTTGACCTCGGAT CAGGTAGGGATACCCGCTGAACTTAAGCATATGAA	*Phoma*	99
47	CM2	CGGTTATAGAGTTAACAAAA CAACTCCCAACCCTTTGTGAACCTTACCTACCGTTGCTTCGGCGGACCGC CCCGGGCGCTGCGTGCCCCGGACCCAAGGCGCCCGCCGGGGACCACACGA ACCCTGTTTAACAAACATGTGTATCCTCTGAGCGAGCCGAAAGGCAACAA	*Trichothecium*	100

		AACAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAA GAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC ATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGC CTGTCCGAGCGTCATTTCAACCCTCGGGCCCCCCCCTTTTCCCCTCGCGG GGGAGGGGGCGGGCCCGGCGTTGGGGCCCAGGCGTCCTCCAAGGGCGCCT GTCCCCGAAACCCAGTGGCGGCCTCGCCGCTGCCTCCTCCGCGTAGTAGC ACAAACCTCGCGGGCGGAAGGCGGCGCGGCCACGCCGTAAAACCCCAAAC TTTTACCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAG CATAGGCCGCCCTGGGTTTCGGGGGACAG GCGCCCTTGGAGGACGCCGGGCCCCAACGCGCCCGCCCCCNCCCCCG CGGGGGAAAGGGGGGGCCCGATTGAAATGACGCTGACAGG CGCCCGCCGAAACTGGCGGCGCGGCGTTCAAAGACGATG ATCATGACTGCAGTTCTTACTTATAATTGTGCGTCT TCGATCCGAACAAGAGTTTTGAATTGAG TTGTTCGGTCGTAGAGAAACATGTTGTA GGTGTGTGGCGGGTCCAGGGCCC		

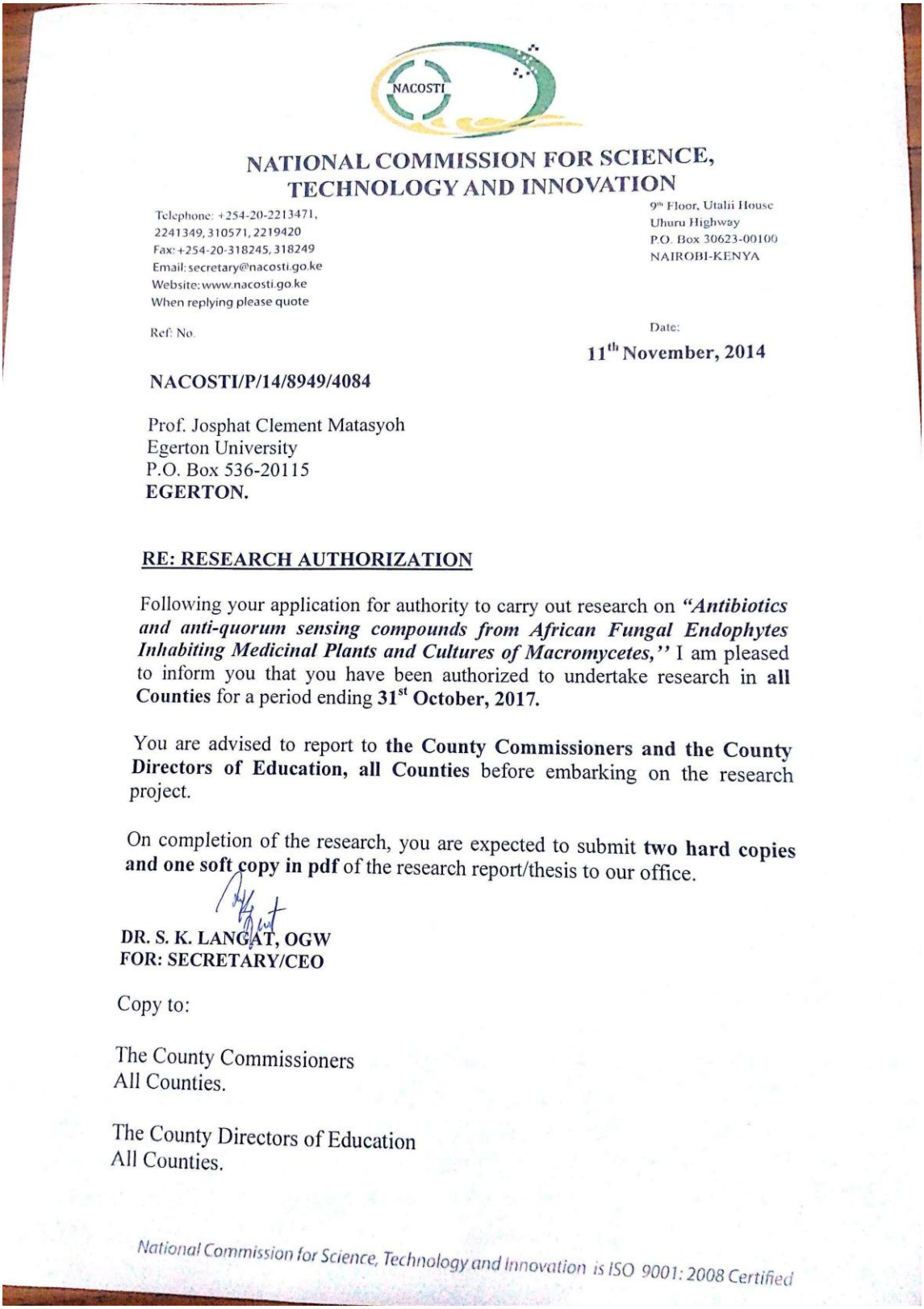
Appendix 4: BLAST search hits of endophytic fungal isolates

Endophyte isolate code	Closely related species	Query coverage	Identity %	Accession number
PF1	*Fusarium* sp.	97%	100%	KT313636.1
PC1	*Fusarium* sp.	100%	99%	KU984712.1
TN2	*Colletotrichum* sp.	97%	99%	JQ341124.1
TN3	*Fusarium* sp.	99%	99%	KU255178.1
PC2	*Colletotrichum* sp.	99%	99%	KP942898.1
TN1	*Fusarium* sp.	98%	99%	HQ130712.1
TI1	*Colletotrichum* sp.	97%	100%	KX058529.1
TA2	*Colletotrichum* sp.	98%	99%	KP942898.1
TI3	*Colletotrichum* sp.	97%	99%	KP942898.1
TI2	*Colletotrichum* sp.	99%	99%	KP942898.1
PF2	*Fusarium* sp.	98%	100%	HQ130712.1
WU1	*Colletotrichum* sp.	97%	99%	KP942898.1
AA1	*Fusarium* sp.	99%	99%	KT313634.1
KA1	*Colletotrichum* sp.	97%	100%	KC860042.1
WU3	*Colletotrichum* sp.	97%	99%	KP942898.1
ES1	*Fusarium* sp.	97%	100%	KT207755.1
PC5	*Colletotrichum* sp.	97%	99%	KP942898.1
TE2	*Phomopsis* sp.	99%	99%	EU002934.1
AG2	*Aspergillus* sp.	98%	99%	KP329760.1

PC4	*Colletotrichum* sp.	99%	99%	KP942898.1
KA2	*Phomopsis* sp.	98%	98%	KF733183.1
WU2	*Fusarium* sp.	99%	99%	KF897851.1
TE1	*Chaetomium* sp.	98%	100%	KF747364.1
TD2	*Pestalotiopsis* sp.	98%	100%	KP747698.1
TD1	*Pestalotiopsis* sp.	97%	100%	KP747698.1
AG1	*Colletotrichum* sp.	97%	99%	JX951175.1
CA4	*Fusarium* sp.	98%	100%	KT313636.1
BA2	*Pestalotiopsis* sp.	99%	100%	AY687875.1
VA1	*Fusarium* sp.	97%	100%	KU204758.1
CA3	*Colletotrichum* sp.	97%	99%	KP942898.1
CA2	*Colletotrichum* sp.	97%	99%	KJ813603.1
CA1	*Colletotrichum* sp.	96%	98%	JQ754029.1
ML3	*Fusarium* sp.	97%	100%	KT313636.1
ML2	*Fusarium* sp.	96%	100%	KU255178.1
TA1	*Colletotrichum* sp.	99%	99%	KP942898.1
ML4	*Fusarium* sp.	98%	100%	KT313636.1
TE4	*Colletotrichum* sp.	99%	100%	KT844640.1
TE5	*Phomopsis* sp.	96%	99%	EU002934.1
WU5	*Colletotrichum* sp.	97%	100%	KT582194.1
ZG1	*Colletotrichum* sp.	98%	99%	KP347577.1
RC1	*Colletotrichum* sp.	98%	100%	KT844640.1

AG3	*Fusarium* sp.	99%	100%	KR528470.1
RC2	*Cladosporium* sp.	98%	100%	KX078479.1
RC1b	*Colletotrichum* sp.	99%	99%	KF772133.1
MW4	*Fusarium* sp.	98%	99%	KT224788.1
MW3	*Phoma* sp.	99%	99%	FJ228201.1
CM2	*Trichothecium* sp.	67%	100%	KT192192.1

Appendix 5: Research Permit



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NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

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Ref: No.

Date:
11th November, 2014

NACOSTI/P/14/8949/4084

Prof. Josphat Clement Matasyoh
Egerton University
P.O. Box 536-20115
EGERTON.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Antibiotics and anti-quorum sensing compounds from African Fungal Endophytes Inhabiting Medicinal Plants and Cultures of Macromycetes,"*** I am pleased to inform you that you have been authorized to undertake research in **all Counties** for a period ending **31st October, 2017.**

You are advised to report to **the County Commissioners and the County Directors of Education, all Counties** before embarking on the research project.

On completion of the research, you are expected to submit **two hard copies and one soft copy in pdf** of the research report/thesis to our office.

DR. S. K. LANGAT, OGW
FOR: SECRETARY/CEO

Copy to:

The County Commissioners
All Counties.

The County Directors of Education
All Counties.

National Commission for Science, Technology and Innovation is ISO 9001: 2008 Certified